AP1 Amine Oxidase Variants

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FIELD OF THE INVENTION

[0002] The present invention relates to the generation of new and novel fumonisin detoxification and fumonisin-derivative detoxification homologues and nucleic acids encoding the same.

BACKGROUND OF THE INVENTION

[0003] Trading of mycotoxin-contaminated agricultural commodities is tightly regulated at both national and international levels. Each year, compliance with these regulations causes the loss of millions of dollars in agricultural produce in the United States alone. Trade sanctions and health effects from mycotoxin-contaminated grains add significantly to the losses (*see, e.g.*, Brown *et al.* (1996) *PNAS USA* 93:14873-14877).

[0004] Accordingly, it is highly desirable to transform various mycotoxins produced by fungal pathogens in crops into inactive compounds which present no human or animal toxicity. This would alleviate important food pollution problems, as well as lessen the costs associated with complying with detecting and destroying mycotoxin-contamination in various crop commodities. Pioneering work in the of construction of nucleic acids for mycotoxin detoxification was done by co-workers of the inventor, *see*, WO 00/20573. The present invention extends this work to the detoxification of fumonisins.

[0005] The term "mycotoxin" generically refers to a number of toxic molecules produced by fungal species, including polyketides and polyketide derived secondary metabolites (such as fumonisins, aflatoxins, sterigmatocystins, alperisins, trichothecenes, fumifungins, and the like). Polyketides are a large, structurally diverse class of secondary metabolites synthesized by bacteria, fungi, and plants and are formed by a polyketide synthase (PKS) through the sequential condensation of small carboxylic acids. *See, e.g.*, Katz and Donadio (1993) *Annu Rev Microbiol* 47:875-912; Brown *et al.* (1996) *PNAS USA*

93:14873-14877; Silva *et al.* (1996) *J Biol Chem* 271:13600-608; Kelkar, I. (1997) *J Biol Chem* 272:1589-94; Busby & Wogan (1985) in *Chemical Carcinogens* (Searle ed., 1985) pp. 945-1136, American Chemical Society, Washington D.C.; Kimura *et al.* (1998) *J Biol Chem* 273:1654-1661.

[0006] Fumonisins are a structurally distinct family of mycotoxins with at least 15 known members, produced by several *Fusarium* species (*see, e.g.*, Scott (1993) *Int J Food Microbiol* 18:257-270 and the references therein). Fumonisins have potential toxic and carcinogenic effects in mammals, and have been associated with a number of animal toxicoses, including equine leukoencephalomalacia and porcine pulmonary edema. Fumonisins mimic sphingolipid precursors inhibiting sphingolipid biosynthesis, a property which is thought to be related to their toxic (*e.g.*, hepatotoxic, renotoxic) and carcinogenic effects. For example, Fumonisin B1 (FB1), the most prevalent of the fumonisins is the diester of propane-1,2,3-tricarboxylic acid and 2-amino-12,16-dimethyl-3,5,10,14,15-pentahydroxyeicosane (empirical formula $C_{34}H_{59}NO_{15}$).

[0007] *Fusarium* infections are widespread among field grown corn (it should be noted that the terms 'corn' and 'maize' are used interchangeably herein), and detectable levels of fumonisins, while more prevalent in corn exhibiting signs of physical damage and infestation, can be found worldwide in food and feed products in the absence of overt symptoms, making it difficult to monitor and eradicate this potentially dangerous toxin. Fumonisins are stable upon exposure to light, and can withstand temperatures commonly used during food processing. For example, following dry milling of corn, fumonisins are found in the resulting bran, germ and flour, and are similarly stable in maize and polenta. However, fumonisins can be hydrolyzed upon treatment with hot alkali solution (i.e., as is performed in some grain treatments/preparations).

[0008] Biological approaches to detoxifying fumonisins have thus far focused on isolating proteins and nucleic acids from naturally occurring organisms capable of metabolizing fumonisins. For example, esterases capable of degrading fumonisins to their de-esterified form, *e.g.*, amino polyol 1 (AP1) and related compounds have been described (*see, e.g.*, US Patent No. 5,716,820, US Patent No. 5,792,931). Similarly, naturally occurring amino polyol amine oxidase (APAO) enzymes capable of oxidatively deaminating AP1 to the 2-oxo derivative of AP1 or its cyclic ketal form have also been described in WO 00/04159 and WO 00/01460. These naturally occurring APAO enzymes have little activity, however, on intact fumonisins.

[0009] The present invention offers new and useful sequences encoding polypeptides with an ability to detoxify fumonisins and fumonisin-derivatives and analogs as well as methods related to detoxification of these mycotoxins. This detoxification is particularly useful in crops, thereby solving each of the problems outlined above, as well as providing a variety of other features which will be apparent upon review.

SUMMARY OF THE INVENTION

[0010] The invention provides novel enzymes useful for detoxification of mycotoxins having primary amine groups, such as fumonisins, fumonisin derivatives and related molecules, including fumonisin hydrolysis products such as amino polyols, *e.g.*, AP1 and similarly configured molecules. For example, fumonisins detoxified by the polypeptides of the invention include fumonisin B1, fumonisin B2, fumonisin B3, fumonisin B4, fumonisin C1, and the like (*e.g.*, structurally similar molecules, etc., such as those having C-2 or C-1 amine groups, etc.). As such, the polypeptides described herein are one set of fumonisin detoxification and fumonisin-derivative detoxification ("FD/FDD") homologue polypeptides. The invention also includes nucleic acids encoding the polypeptides, antibodies to the polypeptides, and uses thereof; data sets containing character strings representing the polynucleotide and polypeptide sequences described herein, and automated systems for using the character strings.

[0011] In one aspect, the invention includes an isolated or recombinant polypeptide with improved fumonisin detoxification characteristics relative to naturally occurring enzymes involved in fumonisin degradation, *e.g.*, a wild type amino polyol amine oxidase enzyme. Generally, such polypeptides are fumonisin amine oxidases. For example, isolated or recombinant polypeptides of the invention have a fumonisin or fumonisin derivative detoxification activity that is at least about 1.5-fold greater than a naturally occurring (or wild-type) enzyme, such as those exemplified by SEQ ID NOs:52, 54, 56, 58, 60, 62, and 64. In some cases, the fumonisin detoxification activity is at least about 2x, in many cases at least about 5x, often at least about 10x, frequently at least about 20x, or more (*e.g.*, 50x, 100x, 250x, 500x, or more) greater than the fumonisin or fumonisin derivative detoxification activity of any of the naturally occurring polypeptides.

[0012] The polypeptides of the invention typically exhibit improved fumonisin or fumonisin derivative detoxification activity, at a pH that is lower than the pH exhibited by any of the naturally occurring enzymes, *e.g.*, represented by SEQ ID NOs: 52, 54, 56, 58, 60, 62 or 64. For example, the polypeptides of the invention exhibit an improved fumonisin detoxification activity at a pH range of between about 5.0 and 7.9. Frequently, the

polypeptides of the invention exhibit the improved fumonisin detoxification activity between about pH 5.5 and pH 7.4. Often, the improved fumonisin detoxification activity is exhibited at a pH between 5.5 and 6.8. In some embodiments, the improved fumonisin detoxification activity exhibits an optimum of about pH 5.5. Polypeptides exhibiting an improved fumonisin detoxification activity at about pH 5.5 are particularly useful for *in vivo* applications where detoxification occurs within the apoplast of a plant cell.

[0013] For example, an improved fumonisin detoxification activity of an FD/FDD polypeptide can be conferred by alterations in the binding of, or alterations in the conversion activity of, a fumonisin, a fumonisin derivative, or a fumonisin-like analog substrate. For example, the polypeptide of the invention having an improved fumonisin detoxification activity can have a higher k_{cat} than any of the naturally occurring enzymes, *e.g.*, exemplified by SEQ ID NOs:52, 54, 56, 58, 60, 62 and 64. Alternatively, or in addition, the polypeptide of the invention has a lower K_M than any of the naturally occurring enzymes described above.

[0014] Additionally, improvements in fumonisin detoxification activity can correlate with increased thermostability relative to a wild type enzyme involved in fumonisin detoxification.

[0015] The polypeptides of the invention having an improved fumonisin detoxification activity are typically at least about 70% identical to SEQ ID NO:50, over a comparison window of at least 125 contiguous amino acids. In one embodiment, the polypeptide comprises a sequence selected from SEQ ID NOs:26-50, SEQ ID NOs:70-72, and SEQ ID NOs:85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107, 109, 111, 113, 115, 117, 119, 121, 123, 125, and 127 or a subsequence or fragment thereof with fumonisin detoxification activity. In some embodiments, the polypeptides are at least about 75% identical to SEQ ID NO:50 over a comparison window of 125 contiguous amino acids. Commonly, the polypeptides are at least about 80%, frequently at least about 85%, often at least about 90%, sometimes at least about 95% or more, *e.g.*, 97%, 98%, or 99% identical to SEQ ID NO:50 over a comparison window of at least 125 contiguous amino acids.

[0016] The invention also includes polypeptides which are substantially identical over at least 125, at least 150, at least 175, at least 200, at least 225, at least 250, at least 275, or at least 300 contiguous amino acids of such a polypeptide with improved fumonisin detoxification activity. For example, in some embodiments, the polypeptides of the invention are substantially identical (*e.g.*, at least about 70%, 75%, 80%, 85%, 90%, 95%, 97%, 98%, or 99% identical) over at least about 125, or more contiguous amino acids of any one of SEQ ID NOs:26- 50, SEQ ID NOs:70- 72, and SEQ ID NOs:85, 87, 89, 91, 93, 95, 97, 99, 101,

103, 105, 107, 109, 111, 113, 115, 117, 119, 121, 123, 125, and 127. In one embodiment, the polypeptides of the invention are identical for at least about 125 contiguous amino acids of any one of SEQ ID NOs:26-50, SEQ ID NOs: 70-72, and SEQ ID NOs:85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107, 109, 111, 113, 115, 117, 119, 121, 123, 125, and 127. For example, a polypeptide of the invention is a protein with an amino acid sequence of any one of SEQ ID NOs:26-50, SEQ ID NOs:70-72, and SEQ ID NOs:85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107, 109, 111, 113, 115, 117, 119, 121, 123, 125, and 127. In other embodiments, the polypeptides of the invention include one or more mutated amino acids, *e.g.*, conservative amino acid substitutions. For example, certain embodiments include, *e.g.*, an alanine residue at position 118, a serine residue at position 136, an asparagine residue at position 193, a phenylalanine residue at position 209, a lysine residue at position 210, an isoleucine residue at position 237, a glutamic acid residue at position 272, a proline residue at position 274, and/or a glutamic acid residue at position 473, wherein the recited positions refer to amino acid positions of the wild type APAO polypeptide sequence (SEQ ID NO: 52). In some embodiments, the polypeptides have an altered glycosylation pattern relative to any one of SEQ ID NOs:52, 54, 56, 58, 60, 62 or 64. An altered glycosylation pattern results from the addition and/or deletion of at least one glycosylation site. Optionally the altered glycosylation site is at an amino acid at positions 201-206 (NDSNQS) (SEQ ID NO: 73).

[0017] In some embodiments the polypeptides of the invention are encoded by polynucleotides selected from among: (a) a polynucleotide sequence of SEQ ID NOs:1-25, SEQ ID NOs:67-69, and SEQ ID NOs:84, 86, 86, 99, 92, 94, 96, 98, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, 120, 122, 124, and 126; (b) a polynucleotide sequence that encodes a polypeptide selected from SEQ ID NOs:26-50, SEQ ID NOs:70-72, and SEQ ID NOs:85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107, 109, 111, 113, 115, 117, 119, 121, 123, 125, and 127; and (c) a complementary sequence of a polynucleotide sequence which hybridizes under highly stringent conditions over substantially the entire length of polynucleotide sequence (a) or (b). In various embodiments, the polypeptide comprises partial or full length sequences (*e.g.*, at least 125, at least 150, at least 175, at least 200, at least 225, at least 250, at least 275, or at least 300 or more amino acids).

[0018] These sequences can be present separately or as components of larger proteins. In various embodiments, the polypeptide comprises about 580, about 585, about 590, about 595, or more (*e.g.*, 596, 597, 598, 599 or 600) contiguous amino acids of the encoded protein. For example, the polypeptides of the invention can be incorporated in fusion proteins. In other embodiments, any polypeptide described above may further include a secretion/localization sequence, *e.g.*, a signal sequence, a membrane localization sequence, an

organelle targeting sequence (*e.g.*, an apoplast targeting sequence or a peroxisome targeting sequence), and the like. For example, a polypeptide of the invention can include a leader sequence, *e.g.*, a leader sequence directing secretion from a cell (such as a plant cell). In the latter instance, the polypeptides typically have an increased fumonisin detoxification activity upon secretion from a cell, relative to any of the polypeptides corresponding to SEQ ID NOs:52, 54, 56, 58, 60, 62 and 64. Similarly, any polypeptide described above may further include a sequence that facilitates purification, *e.g.*, an epitope tag (such as, a FLAG epitope), a polyhistidine tag, a GST fusion, and the like. The polypeptide optionally includes a methionine at the N-terminus. Any polypeptide described above optionally includes one or more modified amino acids, such as a glycosylated amino acid, a PEG-ylated amino acid, a farnesylated amino acid, an acetylated amino acid, a biotinylated amino acid, a carboxylated amino acid, a phosphorylated amino acid, an acylated amino acid, or the like.

[0019] The invention also includes truncated polypeptide versions or fragments of the polypeptides of the invention (*e.g.*, as listed in SEQ ID NOs:26-50, SEQ ID NOs:70-72, and SEQ ID NOs:85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107, 109, 111, 113, 115, 117, 119, 121, 123, 125, and 127) as well as the polynucleotides encoding such truncated polypeptides. The polypeptide fragments can be truncated from either the N-terminus or the C-terminus or from both the N-terminus and the C-terminus. The truncated polypeptides of the invention have the ability to detoxify at least one fumonisin or fumonisin derivative or analog. Additionally, the truncated polypeptides of the invention optionally have the other desirable characteristics of the full length polypeptides of the invention (*e.g.*, as listed in SEQ ID NOs:26-50, SEQ ID NOs:70-72, and SEQ ID NOs:85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107, 109, 111, 113, 115, 117, 119, 121, 123, 125, and 127) as listed and detailed throughout (*e.g.*, improved kinetics over wild-type APAO, enzymatic activity at physiological pH (*e.g.*, pH 5.5), etc.).

[0020] The invention also includes polypeptides which specifically bind polyclonal antisera raised against one or more antigen comprising a polypeptide selected from those comprising the amino acid sequences set forth at SEQ ID NOs:26-50, SEQ ID NOs:70-72, and SEQ ID NOs:85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107, 109, 111, 113, 115, 117, 119, 121, 123, 125, and 127 or fragments thereof. In particular, polypeptides which bind an antisera raised against any amino acid sequence set forth at SEQ ID NOs:26-50, SEQ ID NOs:70-72, and SEQ ID NOs:85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107, 109, 111, 113, 115, 117, 119, 121, 123, 125, and 127, where the antisera is subtracted with one or more proteins selected from one or more (and optionally all) proteins selected from, *e.g.*, those with clone numbers ESP002C2, ESP002C3, ESP003C12, RAT011C1, RAT011C2, RAT011C4

found in publications WO 00/04159 and WO 00/04160, or wild-type APAO from *Exophiala spinifera* ("APAO") (*see*, SEQ ID NOs: 52, 54, 56, 58, 60, 62), or other homologues found in, *e.g.*, GenBank by one of skill in the art.

[0021] The invention also includes antibodies produced by administering one or more polypeptide described above to a mammal, where the antibody does not bind to known FD/FDD, *e.g.*, wild type APAO, homologue encoding sequences selected from, *e.g.*, those corresponding to clone numbers ESP001, ESP002C2, ESP002C3, ESP003C12, RAT011C1, RAT011C2, RAT011C4 found in publications WO 00/04159 and WO 00/04160 (*see*, SEQ ID Nos: 52, 54, 56, 58, 60, 62), or other homologues found in, *e.g.*, a public database such as, *e.g.*, GenBank.

[0022] The invention also includes antibodies which specifically bind a polypeptide comprising a sequence selected from SEQ ID NOs:26-50, SEQ ID NOs:70-72, SEQ ID NOs:85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107, 109, 111, 113, 115, 117, 119, 121, 123, 125, and 127 and . The antibodies are, *e.g.*, polyclonal, monoclonal, chimeric, humanized, single chain, Fab fragments, fragments produced by an Fab expression library, or the like.

[0023] Another aspect of the invention relates to isolated or recombinant nucleic acids encoding fumonisin and fumonisin-derivative detoxification homologues. In particular, the nucleic acids of the invention encode enzymes with fumonisin or fumonisin derivative or analog detoxification activity, and related coding and non-coding nucleic acids. For example, isolated or recombinant nucleic acids of the invention include a polynucleotide sequence that encodes a polypeptide that is at least 70% or more identical to SEQ ID NO:50 over a comparison window of at least 125 amino acids which has a fumonisin detoxification activity or a fumonisin derivative detoxification activity that is at least 1.5-fold greater than any of SEQ ID NOs: 52, 54, 56, 58, 60, 62 or 64 at pH 5.5. Similarly, nucleic acids that encode polypeptides that are at least about 75%, 80%, 85%, 90%, 95%, 97%, 98%, 99% or more identical to SEQ ID NO:50, and or to any of SEQ ID NOs:26-49, SEQ ID NOs:70-72, and SEQ ID NOs:85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107, 109, 111, 113, 115, 117, 119, 121, 123, 125, and 127 are included in the invention. In one embodiment, the nucleic acids encode at least about 125 contiguous amino acids, *e.g.*, at least 150, at least 175, at least 200, at least 225, at least 250, at least 275, at least 300 or more amino acids, such as the full length of any of SEQ ID NOs:26-50, SEQ ID NOs:70-72, and SEQ ID NOs:85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107, 109, 111, 113, 115, 117, 119, 121, 123, 125, and 127. In an embodiment, the nucleic acid is selected from among SEQ ID NOs:1-25, SEQ ID NOs:67-69,

and SEQ ID NOs:84, 86, 86, 99, 92, 94, 96, 98, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, 120, 122, 124, and 126.

[0024] In other embodiments, the nucleic acids of the invention with the desired fumonisin or fumonisin derivative detoxification activity as described above are nucleic acids that hybridize under low stringency or medium stringency conditions to a polynucleotide sequence selected from among: (a) a polynucleotide sequence selected from the group consisting of SEQ ID NOs:1-,25, SEQ ID NOs:67-69 and SEQ ID NOs:84, 86, 86, 99, 92, 94, 96, 98, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, 120, 122, 124, and 126, or a complementary polynucleotide sequence thereof; (b) a polynucleotide sequence encoding a polypeptide selected from SEQ ID NOs:26-50, SEQ ID NOs:70-72, and SEQ ID NOs:85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107, 109, 111, 113, 115, 117, 119, 121, 123, 125, and 127, or a complementary polynucleotide sequence thereof; and (c) a polynucleotide sequence comprising a fragment of (a) or (b), wherein the fragment encodes a polypeptide having at least one fumonisin detoxification activity or at least one fumonisin derivative detoxification activity.

[0025] Isolated and/or recombinant nucleic acids selected from among polynucleotide sequences including SEQ ID NOs:1-25, SEQ ID NOs:67-69, and SEQ ID NOs:84, 86, 86, 99, 92, 94, 96, 98, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, 120, 122, 124, and 126, and complementary polynucleotide sequences thereof; as well as, polynucleotide sequences encoding a polypeptide selected from SEQ ID NOs:26-50, SEQ ID NOs:70-72, and SEQ ID NOs:85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107, 109, 111, 113, 115, 117, 119, 121, 123, 125, and 127, and complementary polynucleotide sequences thereof, are also a feature of the invention. Similarly, a polynucleotide sequence which hybridizes under highly stringent conditions over substantially the entire length of any of the preceding polynucleotide sequences is a feature of the invention. Similarly, fragments of the above which encode a polypeptide having fumonisin or fumonisin derivative detoxification activity are features of the invention.

[0026] The invention also includes an isolated or recombinant nucleic acid comprising a polynucleotide sequence encoding a polypeptide, where the polypeptide comprises an amino acid sequence which is substantially identical over at least 125 contiguous amino acids of any one of SEQ ID NOs:26-50, SEQ ID NOs:70-72 and SEQ ID NOs:85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107, 109, 111, 113, 115, 117, 119, 121, 123, 125, and 127. In various embodiments, the encoded polypeptide is substantially identical over about 150, about 175, about 200, about 225, about 250, about 275, or about 300

or more contiguous amino acid residues or substantially identical variants of any one of the polypeptide sequences listed or encoded by any nucleic acid listed. In other embodiments, the encoded polypeptide is at least about 580 or at least about 595 amino acids in length. In another embodiment, the encoded polypeptide is about 600 amino acids in length. The polypeptides can exist separately or as components of one or more fusion proteins, *e.g.*, including a signaling or leader sequence, a targeting sequence or the like.

[0027] In other embodiments, the nucleic acids of the invention encode polypeptides that detoxify or degrade a fumonisin or a fumonisin-derivative. Other embodiments of the invention include nucleic acids that encode polypeptides which deaminate a fumonisin or a fumonisin-derivative. Preferably, the encoded polypeptide detoxifies, degrades, or deaminates a mycotoxin that is a fumonisin or a fumonisin derivative or analog, *e.g.*, a fumonisin B1, a fumonisin B2, a fumonisin B3, a fumonisin B4, a fumonisin C1, or the like, or structurally related mycotoxins and/or analogs (*see*, Fig. 9 for structures of AP1 and FB1). In one embodiment, the encoded polypeptide is a fumonisin amine oxidase. Optionally, the nucleic acids of the invention encode polypeptides with altered kinetic parameters (*e.g.*, K_{cat}, K_M), such as improved fumonisin or fumonisin derivative detoxification activity at a selected pH, *e.g.*, pH 5.5, relative to a wild-type APAO (amino polyol (AP1) amine oxidase) from, *e.g.*, *Exophiala spinifera.*

[0028] In general, nucleic acids and polypeptides, *e.g.*, proteins, derived by mutation, recursive recombination, or other alterations of the sequences herein are a feature of the invention. Similarly, those produced by recombination, including recursive recombination, are a feature of the invention. Mutation and recombination methods using the nucleic acids described herein are a feature of the invention. For example, one method of the invention includes recombining one or more nucleic acid described above with one or more additional nucleic acids (including, but not limited to those noted herein), the additional nucleic acid encoding a polypeptide with a fumonisin or a fumonisin derivative (or other structurally analogous mycotoxin or mycotoxin derivative) detoxification activity or subsequence thereof. The recombining steps are optionally performed *in vivo* or in vitro. Also included in the invention are a recombinant nucleic acid produced by this method, a cell containing the recombinant nucleic acid, a nucleic acid library produced by this method and a population of cells containing the library.

[0029] The invention also includes a vector comprising any nucleic acid described above, suitable for transforming a prokaryotic or eukaryotic cell, such as a plant. The vector can comprise a plasmid, a cosmid, a phage, or a virus (or virus fragment). In an embodiment,

the vector includes a T-DNA sequence. The vector can be, *e.g.*, an expression vector, a cloning vector, a packaging vector, an integration vector, or the like. For example, an expression vector typically includes a promoter operably linked to the polynucleotide sequence of the invention. Such a promoter can be either constitutive or inducible, and, if desired, is a tissue specific promoter. Frequently, the promoter is heterologous with respect to the polynucleotide of the invention, and is selected to cause sufficient expression of the encoded polypeptide to result in detoxification of fumonisin or a fumonisin derivative in a cell or tissue in which it is expressed. Optionally, any vector of the invention comprises a second polynucleotide sequence encoding a second polypeptide that confers a detectable phenotypic trait upon a cell or organism expressing the polypeptide (*e.g.*, a plant, plant explant, fungus, bacteria, etc.), such as a selectable marker, *e.g.*, herbicide resistance, pest resistance, biocide resistance, fumonisin esterase activity, or a visible marker.

[0030] The invention also includes a cell comprising any nucleic acid (or vector) of the invention, or which expresses any polypeptide noted herein. In one embodiment, the cell expresses a polypeptide encoded by the nucleic acid. Typically, the polynucleotide and/or polypeptide are heterologous to the cell. In some embodiments, the cells incorporating the nucleic acids and/or expressing the polypeptides of the invention are plant cells. Transgenic plants, transgenic plant cells and transgenic plant explants incorporating the nucleic acids of the invention are also a feature of the invention. In some embodiments, the transgenic plants, transgenic plant cells or transgenic plant explants express an exogenous polypeptide with fumonisin detoxification or fumonisin derivative detoxification activity encoded by the nucleic acid of the invention. A seed produced by such a transgenic plant is also a feature of the invention. In such instances, one or more parental codons of the nucleic acid can be substituted with a synonymous codon that is preferentially used by the translation machinery of a plant cell. Alternatively, the cell can be a microorganism cell, such as a bacteria, a fungus or a yeast cell.

[0031] The invention also includes compositions comprising two or more nucleic acids described herein. The composition may comprise a library of nucleic acids, where the library contains at least 5, at least 10, at least 20 or at least 50 or more nucleic acids.

[0032] The invention also includes compositions produced by digesting one or more nucleic acid described herein with a restriction endonuclease, an RNAse, or a DNAse; and, compositions produced by incubating one or more nucleic acid described herein in the presence of deoxyribonucleotide triphosphates and a nucleic acid polymerase, e.g., a thermostable polymerase.

[0033] Methods for producing transgenic organisms (*e.g.*, plants, fungi, bacteria, etc.) comprising a nucleic acid of the invention expressing a polypeptide at an effective level to deaminate fumonisin are also feature of the invention. Additionally, the invention includes methods of reducing pathogenicity of a fungus producing a fumonisin, comprising producing a transgenic cell (also optionally including a plant cell in a plant and optionally wherein the cell comprises a fumonisin esterase encoding polynucleotide linked to a promoter) with a heterologous nucleic acid of the invention operably linked to a promoter and expressing the nucleic acid at a level effective to produce sufficient hydrogen peroxide to reduce fungal infection. Further, the invention includes methods of creating or enhancing disease resistance in a plant by introducing into a plant cell a recombinant expression cassette comprising any of the nucleic acids noted herein operably linked to a promoter that drives expression in a plant, culturing the plant cell under plant cell growing conditions, and regenerating from the plant cell a whole plant, wherein the plant has enhanced or newly created disease resistance.

[0034] Methods for producing the polypeptides of the invention are also included. One such method comprises introducing into a population of cells any nucleic acid described herein, operatively linked to a regulatory sequence effective to produce the encoded polypeptide, culturing the cells in a culture medium to produce the polypeptide, and isolating the polypeptide from the cells or from the culture medium. The nucleic acid may be part of a vector, such as a recombinant expression vector.

[0035] In general, nucleic acids and proteins derived by mutation, recursive recombination, or other alterations of the sequences herein are a feature of the invention. Similarly, those produced by recombination, including recursive recombination, are a feature of the invention. Mutation and recombination methods using the nucleic acids described herein are a feature of the invention. For example, one method of the invention includes recombining one or more nucleic acid described above with one or more additional nucleic acid (including, but not limited to those noted herein), the additional nucleic acid encoding an FD/FDD homologue or subsequence thereof. The recombining steps are optionally performed *in vivo* or in vitro. Also included in the invention are a recombinant nucleic acid produced by this method, a cell containing the recombinant nucleic acid, a nucleic acid library produced by this method and a population of cells containing the library.

[0036] These and other features of the invention will become more fully apparent when the following detailed description is read in conjunction with the accompanying figures.

BRIEF DESCRIPTION OF THE FIGURES

[0037] Figure 1: Kinetic parameters of selected homologues 4F13G12, 4F15A11, 4F15C3, 4F6A11, 4F3B5, 4F2G10, 4F19F2, 4F21C8, 4F22B2, 4F28G1, and a wild-type APAO.

[0038] Figure 2: Kinetic parameters of selected homologues R3H1 and B12.

[0039] Figure 3: Comparison of specific amino acid residue positions between two exemplary homologues.

[0040] Figure 4: Comparison of protein structure between maize polyamine oxidase and exemplary homologues.

[0041] Figure 5: Graph showing degradation activity of exemplary homologue.

[0042] Figure 6: Graph showing substrate specificity of exemplary homologue.

[0043] Figure 7: Graph showing enzymatic activity of exemplary homologue in transgenic maize callus.

[0044] Figure 8: Panels A through D show selected kinetic parameters of exemplary homologues of the invention.

[0045] Figure 9: Illustration of the chemical structures of FB1 and AP1.

[0046] Figure 10: In planta turnover of fumonisin B1 by exemplary homologues of the invention.

[0047] Figure 11: Thin-layer chromatogram (TLC) showing in planta turnover of FB1 in maize embryos by exemplary homologues of the invention.

[0048] Figure 12: Quantitation of TLC showing in planta turnover of FB1 in maize embryos by exemplary homologues of the invention.

[0049] Figure 13: In planta activity of exemplary homologues of the invention in stably transformed maize callus lines.

DETAILED DESCRIPTION OF THE INVENTION

DEFINITIONS

[0050] Before describing the present invention in detail, it is to be understood that this invention is not limited to particular compositions or biological systems, which can, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting. As used in this specification and the appended claims, the singular forms "a", "an" and "the" include plural referents unless the content clearly dictates otherwise. Thus, for example, reference to "a device" includes a combination of two or more such devices, reference to "a gene fusion construct" includes mixtures of constructs, and the like.

[0051] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the invention pertains. Although specific examples of appropriate materials and methods are described herein, any methods and materials similar or equivalent to those described herein can be used in the practice for testing of the present invention.

[0052] In describing and claiming the present invention, the following terminology will be used in accordance with the definitions set out below. The terms "polynucleotide," "nucleotide sequence," and "nucleic acid" are used to refer to a polymer of nucleotides (A,C,T,U,G, etc. or naturally occurring or artificial nucleotide analogues), *e.g.*, DNA or RNA, or a representation thereof, *e.g.*, a character string, etc, depending on the relevant context. A given polynucleotide or complementary polynucleotide can be determined from any specified nucleotide sequence. Similarly, an "amino acid sequence" is a polymer of amino acids (a protein, polypeptide, etc.) or a character string representing an amino acid polymer, depending on context. The terms "protein," "polypeptide," and "peptide" are used interchangeably herein.

[0053] A nucleic acid, protein, peptide, polypeptide, or other component is "isolated" when it is partially or completely separated from components with which it is normally associated (other peptides, polypeptides, proteins (including complexes, *e.g.*, polymerases and ribosomes which may accompany a native sequence), nucleic acids, cells, synthetic reagents, cellular contaminants, cellular components, etc.) in the cell from which it was originally derived. A nucleic acid, polypeptide, or other component is isolated when it is partially or completely recovered or separated from other components of its natural environment such that it is the predominant species present in a composition, mixture, or

collection of components (i.e., on a molar basis it is more abundant than any other individual species in the composition). In preferred embodiments, the preparation contains more than 70%, typically more than 80%, or preferably more than 90% of the isolated species.

[0054] In one aspect, a "substantially pure" or "isolated" nucleic acid (*e.g.,* RNA or DNA), polypeptide, protein, or composition also means where the object species (*e.g.,* nucleic acid or polypeptide) comprises at least about 50, 60, or 70 percent by weight (on a molar basis) of all macromolecular species present. A substantially pure or isolated composition can also comprise at least about 80, 90, or 95 percent (or more) by weight of all macromolecular species present in the composition. An isolated species can also be purified to essential homogeneity (contaminant species cannot be detected in the composition by conventional detection methods) wherein the composition consists essentially of derivatives of a single macromolecular species.

[0055] The term "isolated nucleic acid" may refer to a nucleic acid (*e.g.,* DNA or RNA) that is not immediately contiguous with both of the coding sequences with which it is immediately contiguous (i.e., one at the 5' and one at the 3' end) in the naturally occurring genome of the organism from which the nucleic acid is derived. Thus, this term includes, *e.g.,* a cDNA or a genomic DNA fragment produced by polymerase chain reaction (PCR) or restriction endonuclease treatment, whether such cDNA or genomic DNA fragment is incorporated into a vector, integrated into the genome of the same or a different species than the organism, including, *e.g.,* a virus, from which it was originally derived, linked to an additional coding sequence to form a hybrid gene encoding a chimeric polypeptide, or independent of any other DNA sequences. The DNA may be double-stranded or single-stranded, sense or antisense.

[0056] A nucleic acid or polypeptide is "recombinant" when it is artificial or engineered, or derived from an artificial or engineered protein or nucleic acid. The term "recombinant" when used with reference *e.g.,* to a cell, nucleotide, vector, or polypeptide typically indicates that the cell, nucleotide, or vector has been modified by the introduction of a heterologous (or foreign) nucleic acid or the alteration of a native nucleic acid, or that the polypeptide has been modified by the introduction of a heterologous amino acid, or that the cell is derived from a cell so modified. Recombinant cells express nucleic acid sequences (*e.g.,* genes) that are not found in the native (non-recombinant) form of the cell or express native nucleic acid sequences (*e.g.,* genes) that would be abnormally expressed, under-expressed, or not expressed at all. The term "recombinant nucleic acid" molecule (*e.g.,* DNA or RNA) means, for example, a nucleotide sequence that is not naturally occurring or is made

by the combination (for example, artificial combination) of at least two segments of sequence that are not typically included together, not typically associated with one another, or are otherwise typically separated from one another. A recombinant nucleic acid can comprise a nucleic acid molecule formed by the joining together or combination of nucleic acid segments from different sources and/or a nucleic acid that is artificially synthesized. The term "recombinantly produced" refers to an artificial combination usually accomplished by either chemical synthesis means, recursive sequence recombination of nucleic acid segments or other diversity generation methods (such as, *e.g.,* recursive recombination) of nucleotides, or manipulation of isolated segments of nucleic acids, *e.g.,* by genetic engineering techniques known to those of ordinary skill in the art. "Recombinantly expressed" typically refers to techniques for the production of a recombinant nucleic acid in vitro and transfer of the recombinant nucleic acid into cells *in vivo*, in vitro, or ex vivo where it may be expressed or propagated. A "recombinant polypeptide" or "recombinant protein" usually refers to a polypeptide or protein, respectively, that results from a cloned or recombinant gene or nucleic acid.

[0057] An "antigen" refers to a substance that is capable of eliciting the formation of antibodies in a host or generating a specific population of lymphocytes reactive with that substance. Antigens are typically macromolecules (*e.g.,* proteins and polysaccharides) that are foreign to the host.

[0058] A "subsequence" or "fragment" (which terms may be used interchangeably herein) is any portion of an entire sequence, up to and including the complete sequence.

[0059] Numbering of an amino acid or nucleotide polymer corresponds to numbering of a selected amino acid polymer or nucleic acid when the position of a given monomer component (amino acid residue, incorporated nucleotide, etc.) of the polymer corresponds to the same residue position in a selected reference polypeptide or polynucleotide.

[0060] A vector is a composition for facilitating cell transformation by a selected nucleic acid, or expression of the nucleic acid in the cell. Vectors include, *e.g.,* plasmids, cosmids, viruses, YACs, bacteria, poly-lysine, etc. An "expression vector" is a nucleic acid construct, generated recombinantly or synthetically, with a series of specific nucleic acid elements that permit transcription of a particular nucleic acid in a host cell. The expression vector can be part of a plasmid, virus, or nucleic acid fragment. The expression vector typically includes a nucleic acid to be transcribed operably linked to a promoter.

[0061] The term "heterologous" as used herein describes a relationship between two or more elements which indicates that the elements are not normally found in proximity to one another in nature. Thus, for example, a polynucleotide sequence is "heterologous to" an organism or a second polynucleotide sequence if it originates from a foreign species, or, if from the same species, is modified from its original form. For example, a promoter operably linked to a heterologous coding sequence refers to a coding sequence from a species different from that from which the promoter was derived, or, if from the same species, a coding sequence which is not naturally associated with the promoter (*e.g.* a genetically engineered coding sequence or an allele from a different ecotype or variety). An example of a heterologous polypeptide is a polypeptide expressed from a recombinant polynucleotide in a transgenic organism. Heterologous polynucleotides and polypeptides are forms of recombinant molecules.

[0062] The term "encoding" refers to the ability of a nucleotide sequence to code for one or more amino acids. The term does not require a start or stop codon. An amino acid sequence can be encoded in any one of six different reading frames provided by a polynucleotide sequence and its complement.

[0063] "Substantially an entire length of a polynucleotide or amino acid sequence" refers to at least about 50%, at least about 60%, generally at least about 70%, generally at least about 80%, or typically at least about 90%, 95%, 96%, 97%, 98%, or 99% or more of a length of an amino acid sequence or nucleic acid sequence.

[0064] "Naturally occurring" as applied to an object indicates that the object can be found in nature. For example, a polypeptide or polynucleotide sequence that is present in an organism, including viruses, that can be isolated from a source in nature and which has not been intentionally modified by man in a laboratory is naturally occurring. In one aspect, a "naturally occurring" nucleic acid molecule (*e.g.*, DNA or RNA) is a nucleic acid molecule that exists in the same state as it exists in nature; that is, the nucleic acid molecule is not isolated, recombinant, or cloned.

[0065] The term "immunoassay" includes an assay that uses an antibody or immunogen to bind or specifically bind an antigen. The immunoassay is typically characterized by the use of specific binding properties of a particular antibody to isolate, target, and/or quantify the antigen.

[0066] The term "homology" generally refers to the degree of similarity between two or more structures. The term "homologous sequences" refers to regions in

macromolecules that have a similar order of monomers. When used in relation to nucleic acid sequences, the term "homology" refers to the degree of similarity between two or more nucleic acid sequences (*e.g.,* genes) or fragments thereof. Typically, the degree of similarity between two or more nucleic acid sequences refers to the degree of similarity of the composition, order, or arrangement of two or more nucleotide bases (or other genotypic feature) of the two or more nucleic acid sequences. The term "homologous nucleic acids" generally refers to nucleic acids comprising nucleotide sequences having a degree of similarity in nucleotide base composition, arrangement, or order. The two or more nucleic acids may be of the same or different species or group. The term "percent homology" when used in relation to nucleic acid sequences, refers generally to a percent degree of similarity between the nucleotide sequences of two or more nucleic acids.

[0067] When used in relation to polypeptide (or protein) sequences, the term "homology" refers to the degree of similarity between two or more polypeptide (or protein) sequences or fragments thereof. Typically, the degree of similarity between two or more polypeptide (or protein) sequences refers to the degree of similarity of the composition, order, or arrangement of two or more amino acids of the two or more polypeptides (or proteins). The two or more polypeptides (or proteins) may be of the same or different species or group. The term "percent homology" when used in relation to polypeptide (or protein) sequences, refers generally to a percent degree of similarity between the amino acid sequences of two or more polypeptide (or protein) sequences. The term "homologous polypeptides" or "homologous proteins" generally refers to polypeptides or proteins, respectively, that have amino acid sequences and functions that are similar. Such homologous polypeptides or proteins may be related by having amino acid sequences and functions that are similar, but are derived or evolved from different or the same species using the techniques described herein.

[0068] The term "gene" broadly refers to any segment of DNA associated with a biological function. Genes include coding sequences and/or regulatory sequences required for their expression. Genes also include non-expressed DNA nucleic acid segments that, *e.g.,* form recognition sequences for other proteins (*e.g.,* promoter, enhancer, or other regulatory regions).

[0069] Generally, the nomenclature used hereafter and the laboratory procedures in cell culture, molecular genetics, molecular biology, nucleic acid chemistry, and protein chemistry described below are those well known and commonly employed by those of ordinary skill in the art. Standard techniques, such as described in Sambrook *et al.*, *Molecular Cloning - A Laboratory Manual* (2nd Ed.), Vols. 1-3, Cold Spring Harbor

Laboratory, Cold Spring Harbor, New York, 1989 (hereinafter "Sambrook") and *Current Protocols in Molecular Biology*, F. M. Ausubel *et al.*, eds., Current Protocols, a joint venture between Greene Publishing Associates, Inc. and John Wiley & Sons, Inc. (supplemented through 2003) (hereinafter "Ausubel"), are used for recombinant nucleic acid methods, nucleic acid synthesis, cell culture methods, and transgene incorporation, *e.g.*, electroporation, injection, and lipofection. Generally, oligonucleotide synthesis and purification steps are performed according to specifications. The techniques and procedures are generally performed according to conventional methods in the art and various general references which are provided throughout this document. The procedures therein are believed to be well known to those of ordinary skill in the art and are provided for the convenience of the reader.

[0070] As used herein, an "antibody" refers to a protein comprising one or more polypeptides substantially or partially encoded by immunoglobulin genes or fragments of immunoglobulin genes. The recognized immunoglobulin genes include the kappa, lambda, alpha, gamma, delta, epsilon and mu constant region genes, as well as myriad immunoglobulin variable region genes. Light chains are classified as either kappa or lambda. Heavy chains are classified as gamma, mu, alpha, delta, or epsilon, which in turn define the immunoglobulin classes, IgG, IgM, IgA, IgD and IgE, respectively. A typical immunoglobulin (*e.g.*, antibody) structural unit comprises a tetramer. Each tetramer is composed of two identical pairs of polypeptide chains, each pair having one "light" (about 25 kD) and one "heavy" chain (about 50-70 kD). The N-terminus of each chain defines a variable region of about 100 to 110 or more amino acids primarily responsible for antigen recognition. The terms variable light chain (VL) and variable heavy chain (VH) refer to these light and heavy chains, respectively. Antibodies exist as intact immunoglobulins or as a number of well characterized fragments produced by digestion with various peptidases. Thus, for example, pepsin digests an antibody below the disulfide linkages in the hinge region to produce F(ab)'2, a dimer of Fab which itself is a light chain joined to VH-CH1 by a disulfide bond. The F(ab)'2 may be reduced under mild conditions to break the disulfide linkage in the hinge region thereby converting the F(ab)'2 dimer into an Fab' monomer. The Fab' monomer is essentially an Fab with part of the hinge region (*see*, Paul, W.E., ed. (1993) *Fundamental Immunology*, Raven Press, N.Y., for a more detailed description of other antibody fragments). While various antibody fragments are defined in terms of the digestion of an intact antibody, one of skill will appreciate that such Fab' fragments may be synthesized de novo either chemically or by utilizing recombinant DNA methodology. Thus, the term antibody, as used herein also includes antibody fragments either produced by the modification of whole antibodies or synthesized *de novo* using recombinant DNA methodologies. Antibodies include single chain antibodies, including single chain Fv (sFv) antibodies in which a variable

heavy and a variable light chain are joined together (directly or through a peptide linker) to form a continuous polypeptide.

[0071] The term "plant" includes whole plants, shoot vegetative organs/structures (*e.g.* leaves, stems and tubers), roots, flowers and floral organs/structures (*e.g.* bracts, sepals, petals, stamens, carpels, anthers and ovules), seed (including embryo, endosperm, and seed coat) and fruit (the mature ovary), plant tissue (*e.g.* vascular tissue, ground tissue, and the like) and cells (*e.g.* guard cells, egg cells, trichomes and the like), and progeny of same. The class of plants that can be used in the methods of the invention is generally as broad as the class of higher and lower plants, including angiosperms (monocotyledonous and dicotyledonous plants), gymnosperms, ferns, and multicellular algae. It includes plants of a variety of ploidy levels, including aneuploid, polyploid, diploid, haploid and hemizygous. In some optional embodiments, the class of plants capable of use in some methods of the invention is generally as broad as the class of higher and lower plants amenable to transformation techniques (*e.g.*, chosen from such groups listed above).

[0072] As used herein, the term "fumonisin" encompasses all structural variants and analogs (also referred to herein as "structurally related mycotoxins" or "structurally related fumonisins") of fumonisin. The term includes fumonisin variants and analogs capable of being, or expected of being, degraded (either wholly or in part) by the activity of an enzyme/polypeptide/polypeptide fragment/etc. of the present invention. Typically, such degradation is to be taken to mean deamination of the fumonisin or fumonisin variant/analog. Such structurally related fumonisins have a chemical structure related to fumonisin and include, *e.g.*, AP1, AAL toxin, fumonisin B1, fumonisin B2, fumonisin B3, fumonisin B4, and fumonisin C1 (as well as their derivatives (*see, supra*)). Furthermore, other mycotoxins which have similar chemical structures to fumonisin, including ones that are synthetically constructed and/or ones which contain a C-2 or C-1 amine groups and one or more adjacent hydroxyl groups are included within the term. The term "fumonisin derivative" relates to any chemically and/or structurally modified fumonisin, such as the hydrolyzed form of fumonisin (i.e., AP1) whether such modification is done naturally (*e.g.*, through enzymatic action of a naturally occurring organism) or through human action.

[0073] "Detoxification" of a fumonisin or of a fumonisin-derivative or analog means any modification of the fumonisin or of the fumonisin-derivative or analog molecule that causes a decrease in that molecule's toxicity from about a 1% or less decrease in toxicity to about a 5%, about 10%, about 25%, about 50%, about 75%, about 90%, about 95%, about 99%, or more decrease in toxicity. Detoxification can be the result of any number of changes

to the fumonisin or fumonisin-derivative or analog including, but not limited to the addition or deletion of a chemical moiety, the cleavage of a chemical bond, oxidation, reduction, etc. Here, in typical embodiments, the detoxification results from (and is taken to typically mean) a deamination of the fumonisin (or derivative or analog). Detoxification is also described herein by "degradation," "neutralization," and/or "modification."

[0074] A variety of additional terms are defined or otherwise characterized herein.

POLYNUCLEOTIDES

[0075] The invention provides polynucleotides that encode transcription and/or translation products that are subsequently spliced to ultimately produce functional fumonisin degrading polypeptides. Splicing can be accomplished in vitro or *in vivo*, and can involve cis or trans splicing. The substrate for splicing can be polynucleotides (*e.g.*, RNA transcripts) or polypeptides. An example of cis splicing of a polynucleotide is when an intron inserted into a coding sequence is removed and the two flanking exon regions are spliced to generate a functional polypeptide encoding sequence. An example of trans splicing is when a polynucleotide is encrypted by separating the coding sequence into two or more fragments that can be separately transcribed and then spliced to form the full-length fumonisin detoxification encoding sequence. The use of a splicing enhancer sequence (which can be introduced into a construct of the invention) can facilitate splicing either in cis or trans. Cis and trans splicing of polypeptides are described in more detail elsewhere herein. More detailed description of cis and trans splicing can be found in US Patent Nos. 6,365,377 and 6,531,316.

[0076] Thus, some polynucleotides of the invention do not directly encode a full-length fumonisin detoxification polypeptide, but rather encode a fragment or fragments of a fumonisin detoxification polypeptide. These fumonisin detoxification polynucleotides can be used to express a functional fumonisin detoxification polypeptide through a mechanism involving splicing, where splicing can occur at the level of polynucleotide (*e.g.*, intron/exon) and/or polypeptide (*e.g.*, intein/extein). This can be useful, for example, in controlling expression of fumonisin detoxification activity, since functional fumonisin detoxification polypeptide will only be expressed if all required fragments are expressed in an environment that permits splicing processes to generate functional product. In another example, introduction of one or more insertion sequences into a fumonisin detoxification polynucleotide can facilitate recombination with a low homology polynucleotide; use of an intron or intein for the insertion sequence facilitates the removal of the intervening sequence, thereby restoring function of the encoded variant.

[0077] The polypeptides of the invention and their encoding nucleic acids fill an unmet need by providing fumonisin detoxification enzymes that can detoxify fumonisins (*e.g.*, FB1) and/or fumonisin-derivatives (*e.g.*, AP1) or fumonisin analogs. In some embodiments, the polynucleotides of the invention encode enzymes (or fragments of such) capable of degrading fumonisin (and its derivatives/analogs at plant apoplast pH (*e.g.*, pH 5.5). These aspects of the present invention are useful in, *e.g.*, construction of transgenic crop plants that are resistant to or more tolerant of fumonisin and/or fumonisin derivatives and are able to degrade or neutralize fumonisin and/or fumonisin derivatives thus making the plants safer for human and animal consumption. Additionally, aspects of the present invention are useful in applications to foodstuffs, notably grains, etc., to degrade/neutralize fumonisin.

Fumonisin Detoxification Homologue Sequences

[0078] The invention provides isolated or recombinant novel fumonisin detoxification polypeptides and homologues thereof, and isolated or recombinant polynucleotides encoding the polypeptides. The invention also provides truncated versions of the isolated or recombinant fumonisin detoxification polypeptides (*e.g.*, *see*, SEQ ID NO:21 and SEQ ID NO:46 (truncated version of R3H1)) as well as polynucleotides encoding such truncated polypeptides (*e.g.*, fragments of polynucleotides which encode functional fumonisin detoxification polypeptides). The truncated fumonisin detoxification polypeptides can be truncated from either the C-terminus or the N-terminus or from both the N-terminus and the C-terminus. The truncated polypeptides of the invention optionally display the ability to detoxify at least one fumonisin and/or at least one fumonisin-derivative. Additionally, the truncated polypeptides of the invention optionally have the other capabilities of the non-truncated polypeptides of the invention as are listed and detailed throughout the present specification (*e.g.*, improved kinetics over wild-type APAO, enzymatic activity at physiological pH (*e.g.*, pH 5.5), etc.). Some of the fumonisin detoxification homologues of the invention include embodiments comprising fumonisin detoxifying and/or fumonisin-derivative detoxifying ability at a pH range of between 5.0 and 7.4, of between 5.0 and 7.0, of between 5.0 and 6.5, of between 5.0 and 6.0, or of between 5.0 and 5.5.

[0079] In some aspects, the current invention comprises an isolated or recombinant nucleic acid with a polynucleotide sequence encoding a polypeptide that is at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99%, or at least about 99.5%, or more identical to (or which is substantially identical to, or comprises) to one or more of SEQ ID NOs:26-50, SEQ ID NOs:70-72, and SEQ ID NOs:85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107, 109, 111, 113, 115, 117, 119, 121, 123, 125, and 127 (*e.g.*, to SEQ

ID NO:50) over a comparison window of at least 100, at least 125, at least 150, at least 175, at least 200, at least 225, at least 250, at least 275, or at least 300 contiguous amino acids (or complementary polynucleotide sequences thereof) wherein the polypeptide has a fumonisin detoxification activity or a fumonisin-derivative detoxification activity that is at least 1.5x, at least 2x, at least 5x, at least 10x, at least 15x, at least 20x, or at least 25x or more greater than any of the polypeptides corresponding to SEQ ID NOs:51-64.

[0080] Optionally, the above polynucleotide encodes a polypeptide that displays increased FD/FDD activity at pH 5.5 or has an optimum pH lower than that for the polypeptides encoded by SEQ ID NOs:51-64. Optionally, the above polynucleotide encodes a polypeptide that displays greater thermostability than that of any polypeptide encoded by SEQ ID NOs:51-64 and/or optionally has increased FD/FDD activity upon secretion from a eukaryotic cell (*e.g.*, a plant cell) relative to that activity of any polypeptide encoded by SEQ ID NOs:51-64. In some embodiments, the polynucleotide encodes a polypeptide which comprises a leader sequence that directs secretion of the polypeptide from a plant cell (*e.g.*, an apoplast targeting sequence, a peroxisomal targeting sequence, etc.), alternately and/or additionally, the polynucleotide encodes a polypeptide which comprises a polypeptide purification sequence. In yet other aspects, the invention comprises a nucleic acid comprising a unique subsequence in a nucleic acid selected from SEQ ID NOs:1-25, SEQ ID NOs:67-69, and SEQ ID NOs:84, 86, 86, 99, 92, 94, 96, 98, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, 120, 122, 124, and 126, which is unique as compared to a nucleic acid comprising any one of SEQ ID NOs:51, 53, 55, 57, 59, 61, 63, or to the nucleic acid encoding any of SEQ ID NOs: 52, 54, 56, 58, 60, 62, 64.

[0081] Furthermore, in some embodiments, such above polynucleotide encodes a polypeptide that is at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99%, or at least about 99.5%, or more identical to, or is substantially identical to, or is chosen from any one or more of SEQ ID NOs:26-50, SEQ ID NOs:70-72, and SEQ ID NOs:85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107, 109, 111, 113, 115, 117, 119, 121, 123, 125, and 127, or that is chosen from the group comprising SEQ ID NOs:1-25, SEQ ID NOs:67-69, and SEQ ID NOs:84, 86, 86, 99, 92, 94, 96, 98, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, 120, 122, 124, and 126. The FD/FDD activity of a polypeptide encoded by such an above polynucleotide is, in typical embodiments, the ability to deaminate fumonisin and/or fumonisin derivatives (*e.g.*, fumonisin B1, fumonisin B2, fumonisin B3, fumonisin B4, fumonisin C1, or a structural analog, etc.), i.e., the polypeptide encoded by the polynucleotide is a fumonisin amine oxidase. In yet other embodiments, the

above polynucleotide encodes a polypeptide which displays one or more of: a k_{cat} (optionally at pH5.5) greater than or higher than the k_{cat} of any of the polypeptides encoded by SEQ ID NOs:51-64; a K_M value (optionally at pH 5.5) lower than the K_M value of any of the polypeptides encoded by SEQ ID NOs:51-64; or a k_{cat} / K_M value higher than, or greater than the k_{cat} / K_M value of any of the polypeptides encoded by SEQ ID NOs:51-64 when catalyzing a fumonisin or fumonisin-derivative reaction (*e.g.,* a fumonisin deamination reaction).

[0082] In some embodiments of the invention, the above polynucleotides encode polypeptides comprising variants wherein one or more amino acid has been mutated. In yet other embodiments, the above polynucleotides encode polypeptides wherein the polypeptide comprises an alanine residue at position 118, a serine residue at position 136, a phenylalanine reside at position 209, a lysine residue at position 210, an isoleucine residue at position 237, a glutamic acid residue at position 272, a proline residue at position 274, and a glutamic acid residue at position 473, wherein the recited positions refer to amino acid positions of the polypeptide of the wild type APAO enzyme (SEQ ID NO: 52). In yet other embodiments, the above polynucleotide encodes a polypeptide comprises an aspartic acid residue at position 193. Some embodiments of the current invention also comprise polynucleotides encoding polypeptides with an altered glycosylation site.

[0083] In some embodiments, the polynucleotide of the invention comprises a promoter operably linked to the polynucleotide, wherein the promoter is optionally tissue-specific and/or wherein such construct comprises a vector (*e.g.,* wherein the vector comprises a first polynucleotide sequence comprising the promoter operably linked to the polynucleotide of the invention and a second polynucleotide encoding a second polypeptide that confers a detectable phenotypic trait on a cell or organism expressing the second polypeptide at an effective level, such as herbicide resistance, pest resistance, a visible marker, etc.; or wherein the vector comprises a T-DNA; or wherein the vector is a plant transformation vector). Furthermore, the optional promoter is optionally heterologous with respect to the polynucleotide and is optionally effective to cause sufficient expression of the encoded polypeptide to cause detoxification (*e.g.,* typically through deamination) of a fumonisin and/or a fumonisin-derivative. In other embodiments, the polynucleotides of the invention comprise a selectable marker and/or function as a selectable marker. Some embodiments of the invention comprise polynucleotides wherein a parental codon of the polynucleotide sequence has been replaced by a synonymous codon that is preferentially used in a plant relative to the parental codon.

[0084] In yet other embodiments the invention comprises an isolated or recombinant nucleic acid which encodes a polypeptide that (optionally at pH 5.5) has a fumonisin detoxification and/or a fumonisin-derivative detoxification (*e.g.*, fumonisin deamination) activity that is at least 1.5x, at least 2x, at least 5x, at least 10x, at least 15x, at least 20x, or at least 25x or more greater than any of the polypeptides corresponding to SEQ ID NOs:51-64 (or a complementary polynucleotide sequence thereof), and wherein the polynucleotide sequence hybridizes under low or medium stringency conditions to a polynucleotide sequence selected from: a polynucleotide sequence selected from SEQ ID NOs:1-25, SEQ ID NOs:67-69, and SEQ ID NOs:84, 86, 86, 99, 92, 94, 96, 98, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, 120, 122, 124, and 126 (or a complementary polynucleotide sequence thereof); a polynucleotide sequence encoding a polypeptide selected from SEQ ID NOs:26-50, SEQ ID NOs:70-72, and SEQ ID NOs:85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107, 109, 111, 113, 115, 117, 119, 121, 123, 125, and 127 (or a complementary polynucleotide sequence thereof); and a polynucleotide sequence comprising a fragment of any of the above wherein such fragment encodes a polypeptide having at least one fumonisin detoxification or fumonisin-derivative detoxification activity. In other embodiments the invention comprises an isolated or recombinant nucleic acid selected from: a) a polynucleotide sequence from the groups consisting of SEQ ID NOs:1-25, SEQ ID NOs: 67-69, and SEQ ID NOs:84, 86, 86, 99, 92, 94, 96, 98, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, 120, 122, 124, and 126 (or a complementary polynucleotide sequence thereof); b) a polynucleotide sequence encoding a polypeptide selected from SEQ ID NOs:26-50, SEQ ID NOs:70-72, and SEQ ID NOs:85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107, 109, 111, 113, 115, 117, 119, 121, 123, 125, and 127 (or a complementary polynucleotide sequence thereof); c) a polynucleotide sequence which hybridizes under highly stringent conditions over substantially the entire length of a polynucleotide sequence encoding a polypeptide selected from a or b (or a complementary polynucleotide sequence thereof); and a polynucleotide sequence comprising a fragment of a, b, or c, wherein the fragment encodes a polypeptide having at least one fumonisin detoxification or fumonisin-derivative detoxification activity (*e.g.*, a fumonisin deamination activity).

[0085] Some fumonisin detoxification polypeptides of the present invention exhibit an ability to detoxify fumonisin and fumonisin-derivatives (*e.g.*, FB1 and AP1). In contrast to known naturally occurring APAO enzymes (*e.g.*, ESP002C2, ESP002C3, ESP003C12, RAT011C1, RAT011C2, RAT011C4), which exhibit little activity at plant apoplast pH (5.5) or towards FB1 (the native substrate of naturally occurring APAO being hydrolyzed FB1), some fumonisin detoxification polypeptides of the present invention exhibit activity at pH 5.5, as well as activity towards FB1. As shown in Figures 1 and 2, exemplary polypeptides of

the invention exhibit at least a three-fold or more increase in enzymatic activity with respect to either FB1 and/or AP1 as compared to wild-type APAO. Some fumonisin detoxification polypeptides of the present invention exhibit an ability to degrade, detoxify, or neutralize, etc., fumonisin and/or fumonisin-derivatives at a range of pH levels, i.e., in addition to their ability to do so at pH 5.5.

[0086] Of the fumonisin detoxification molecules of the present invention, the amino acid sequences of 2 exemplary homologues are compared in Figure 3. As is shown in Figure 3, homologues R3H1 and B12 share 8 amino acid changes from wild-type APAO, namely: Ala 118, Ser 136, Phe 209, Lys 210, Ile 237, Glu 272, Pro 274, and Glu 473, i.e., the amino acid in position 118 of the wild-type APAO enzyme (SEQ ID NO: 52) is changed to alanine, etc. Additionally, homologue R3H1 contains a unique asparagine to aspartic acid change at position 193. The numbering of the above sequence residues is based upon the full-length sequence (i.e., the numbers correspond to the residues' positions in the sequence prior to truncation). As seen in, *e.g.,* Figure 2, homologue R3H1 displays high k_{cat} values that are believed to correlate with the unique amino acid change at position 193 (and which is greater than that of wild-type). Figure 4 illustrates the location of several amino acid changes of R3H1 and B12 homologues as compared to maize polyamine oxidase. As can be seen from the figure, several of the R3H1 and B12 changes occur in a putative substrate binding region.

[0087] Figures 5, 6, and 7 further illustrate the characteristics of homologue R3H1. Figure 5 illustrates the time course of FB1 degradation at pH 5.5 by R3H1 as compared to FB1 degradation at pH 5.5 by wild-type amine oxidase (SEQ ID NO:52). The R3H1 homologue degrades a greater amount of FB1 (as measured in parts per million) than either a blank control or the wild-type amine oxidase. Figure 6 illustrates the substrate specificity of homologue R3H1 (i.e., as against putrescine, lysine, serotonin, spermine, or FB2) at pH 7.4. As shown, R3H1 has high specificity for fumonisins (*e.g.,* FB2) as opposed to non-fumonisins. Figure 7 illustrates homologue R3H1's degradation activity in transgenic maize calluses when expressed in cytosol or fused to a signal sequence at pH 7.5 and 5.5 as compared against the wild-type APAO (SEQ ID NO:52).

[0088] Other embodiments of the invention include the fumonisin detoxification homologues comprising mutations in and/or about amino acid residues 201 through 206 (i.e., NDSNQS (SEQ ID NO:73)), thus including mutations of any glycosylation sites within such area. Additionally, other embodiments of the invention include fumonisin detoxification homologues that have had mutations introduced (*e.g.,* mutated through use of oligos, etc.) at amino acid positions 201 and 204.

Making Polynucleotides

[0089] Polynucleotides and oligonucleotides of the invention can be prepared by standard solid-phase methods, according to known synthetic methods. Typically, fragments of up to about 100 bases are individually synthesized, then joined (*e.g.,* by enzymatic or chemical ligation methods, or polymerase mediated recombination methods) to form essentially any desired continuous sequence. For example, the polynucleotides and oligonucleotides of the invention encoding fumonisin detoxification polypeptides can be prepared by chemical synthesis using, *e.g.,* the classical phosphoramidite method described by, *e.g.,* Beaucage *et al.* (1981) *Tetrahedron Lett* 22:1859-69, or the method described by Matthes *et al.* (1984) *EMBO J* 3:801-05, *e.g.,* as is typically practiced in automated synthetic methods. According to the phosphoramidite method, oligonucleotides are synthesized, *e.g.,* in an automatic DNA synthesizer, purified, annealed, ligated and cloned in appropriate vectors.

[0090] In addition, essentially any nucleic acid can be custom ordered from any of a variety of commercial sources, such as The Midland Certified Reagent Company (mcrc@oligos.com), The Great American Gene Company (www.genco.com), ExpressGen Inc. (www.expressgen.com), Operon Technologies Inc. (Alameda, CA) and many others. Similarly, peptides and antibodies can be custom ordered from any of a variety of sources, such as PeptidoGenic (pkim@ccnet.com), HTI Bio-products, inc. (www.htibio.com), BMA Biomedicals Ltd. (U.K.), BioSynthesis, Inc., and many others.

[0091] Certain polynucleotides of the invention can also be obtained by screening cDNA libraries (*e.g.,* libraries generated by recombining homologous nucleic acids as in typical recursive recombination methods) using oligonucleotide probes which can hybridize to, or PCR-amplify, polynucleotides which encode the fumonisin detoxification homologue polypeptides and fragments of those polypeptides. Procedures for screening and isolating cDNA clones are well known to those of skill in the art. Such techniques are described in, for example, Sambrook and Ausubel. Some polynucleotides of the invention can be obtained by altering a naturally occurring backbone, *e.g.,* by mutagenesis or oligonucleotide recombination. In other cases, such polynucleotides can be made in silico or through oligonucleotide recombination methods as described in the references cited herein.

[0092] As described in more detail herein, the polynucleotides of the invention include some sequences which encode mature fumonisin detoxification polypeptide homologues of the sequences and sequences complementary to these sequences, and novel fragments of coding sequence and complements thereof. The polynucleotides can be in the

form of RNA or in the form of DNA, and include mRNA, cRNA, synthetic RNA and DNA, and cDNA. The polynucleotides can be double-stranded or single-stranded, and if single-stranded, can be the coding strand or the non-coding (anti-sense, complementary) strand. The polynucleotides optionally include the coding sequence of a fumonisin detoxification homologue (i) in isolation, (ii) in combination with additional coding sequence, so as to encode, *e.g.*, a fusion protein, a pre-protein, a prepro-protein, or the like, (iii) in combination with non-coding sequences, such as introns, control elements such as a promoter, a terminator element, or 5' and/or 3' untranslated regions effective for expression of the coding sequence in a suitable host, and/or (iv) in a vector or host environment in which fumonisin detoxification homologue coding sequence is a heterologous gene. Sequences can also be found in combination with typical compositional formulations of nucleic acids, including in the presence of carriers, buffers, adjuvants, excipients and the like.

Using Polynucleotides

[0093] The polynucleotides and fragments thereof of the invention have a variety of uses in, for example: recombinant production (i.e., expression) of the fumonisin detoxification homologue polypeptides of the invention; as transgenes (*e.g.*, to confer fumonisin detoxification ability in transgenic plants); as immunogens; as diagnostic probes for the presence of complementary or partially complementary nucleic acids (including for detection of natural fumonisin detoxification coding nucleic acids); as substrates for further reactions, *e.g.*, recursive recombination reactions or mutation reactions to produce new and/or improved fumonisin detoxification homologues, and the like.

EXPRESSION OF POLYPEPTIDES

[0094] In accordance with the present invention, polynucleotide sequences which encode mature fumonisin detoxification homologues, fragments of fumonisin detoxification proteins, related fusion proteins, or functional equivalents thereof, collectively referred to herein as "fumonisin detoxification or fumonisin-derivative detoxification homologue polypeptides," or "FD/FDD homologue polypeptides" or, more simply, "fumonisin detoxification or fumonisin-derivative detoxification homologues," or "FD/FDD homologues," are used in recombinant DNA molecules that direct the expression of the FD/FDD homologues in appropriate host cells. Due to the inherent degeneracy of the genetic code, other nucleic acid sequences which encode substantially the same or functionally equivalent amino acid sequences are also used to synthesize, clone and express the FD/FDD homologues.

Modified Coding Sequences

[0095] As will be understood by those of skill in the art, it can be advantageous to modify a coding sequence to enhance its expression in a particular host. The genetic code is redundant with 64 possible codons, but most organisms preferentially use a subset of these codons. The codons that are utilized most often in a species are called optimal codons, and those not utilized very often are classified as rare or low-usage codons (*see, e.g.,* Zhang, S. P. *et al.* (1991) *Gene* 105:61-72). Codons can be substituted to reflect the preferred codon usage of the host, a process called "codon optimization" or "controlling for species codon bias."

[0096] Optimized coding sequences containing codons preferred by a particular prokaryotic or eukaryotic host (*see, e.g.,* Murray, E. *et al.* (1989) *Nuc Acids Res* 17:477-508) can be prepared, for example, to increase the rate of translation or to produce recombinant RNA transcripts having desirable properties, such as a longer half-life, as compared with transcripts produced from a non-optimized sequence. Translation stop codons can also be modified to reflect host preference. For example, preferred stop codons for *S. cerevisiae* and mammals are UAA and UGA respectively. The preferred stop codon for monocotyledonous plants is UGA, whereas insects and *E. coli* prefer to use UAA as the stop codon (Dalphin, M. E. *et al.* (1996) *Nuc Acids Res* 24:216-218).

[0097] The polynucleotide sequences of the present invention can be engineered in order to alter the FD/FDD homologue coding sequence of the invention for a variety of reasons, including but not limited to, alterations which modify the cloning, processing and/or expression of the gene product. For example, alterations may be introduced using techniques which are well known in the art, *e.g.,* site-directed mutagenesis, to insert new restriction sites, to alter glycosylation patterns, to change codon preference, to introduce splice sites, etc. Further details regarding silent and conservative substitutions are provided below.

Vectors, Promoters and Expression Systems

[0098] The present invention also includes recombinant constructs comprising one or more of the nucleic acid sequences, as broadly described above. The constructs comprise a vector, such as a plasmid, a cosmid, a phage, a virus, a bacterial artificial chromosome (BAC), a yeast artificial chromosome (YAC), and the like, into which a nucleic acid sequence of the invention (*e.g.,* one which encodes for a polypeptide having FD/FDD ability or a fragment thereof) has been inserted, in a forward or reverse orientation. In a preferred aspect of this embodiment, the construct further comprises regulatory sequences, including, for

example, a promoter, operably linked to the sequence. Large numbers of suitable vectors and promoters are known to those of skill in the art, and are commercially available.

[0099] The invention further provides vectors with stacked traits, i.e., vectors that encode an FD/FDD ability and that also include a second polynucleotide sequence encoding, *e.g.,* a second polypeptide that confers a detectable phenotypic trait upon a cell or organism (*e.g.,* a plant, plant explant, fungus, bacteria, etc.) expressing the second polypeptide at an effective level. The detectable phenotypic trait can function as a selectable marker, *e.g.,* by conferring herbicide resistance, pest resistance, or providing some sort of visible marker. Other examples of such "stacked traits" include, *e.g.,* fumonisin modification activity, chitinase activity, antifungal activity, mycotoxin detoxification activity, herbicidal activity pesticidal activity, nematicidal activity, fumonisin esterase activity, etc.

[0100] In certain embodiments the nucleic acid sequences of the present invention can be stacked with any combination of polynucleotide sequences of interest in order to create plants with a desired phenotype. For example, the polynucleotides of the present invention may be stacked with any other polynucleotides of the present invention, or with other genes implicated in fumonisin detoxification metabolic pathways. The combinations generated can also include multiple copies of any one of the polynucleotides of interest. The polynucleotides of the present invention can also be stacked with any other gene or combination of genes to produce plants with a variety of desired trait combinations including but not limited to traits desirable for animal feed such as high oil genes (*e.g.,* U.S. Patent No. 6,232,529); balanced amino acids (*e.g.* hordothionins (U.S. Patent Nos. 5,990,389; 5,885,801; 5,885,802; and 5,703,409); barley high lysine (Williamson et al. (1987) *Eur. J. Biochem.* 165:99-106; and WO 98/20122); and high methionine proteins (Pedersen et al. (1986) *J. Biol. Chem.* 261:6279; Kirihara et al. (1988) *Gene* 71:359; and Musumura et al. (1989) *Plant Mol. Biol.* 12: 123)); increased digestibility (*e.g.,* modified storage proteins (U.S. Application Serial No. 10/053,410, filed November 7, 2001); and thioredoxins (U.S. Application Serial No. 10/005,429, filed December 3, 2001)), the disclosures of which are herein incorporated by reference. The polynucleotides of the present invention can also be stacked with traits desirable for insect, disease or herbicide resistance (*e.g., Bacillus thuringiensis* toxic proteins (U.S. Patent Nos. 5,366,892; 5,747,450; 5,737,514; 5723,756; 5,593,881; Geiser et al (1986) *Gene* 48:109); lectins (Van Damme et al. (1994) *Plant Mol. Biol.* 24:825); fumonisin detoxification genes (U.S. Patent No. 5,792,931); avirulence and disease resistance genes (Jones et al. (1994) *Science* 266:789; Martin et al. (1993) *Science* 262:1432; Mindrinos et al.

(1994) *Cell* 78:1089); acetolactate synthase (ALS) mutants that lead to herbicide resistance such as the S4 and/or Hra mutations; inhibitors of glutamine synthase such as phosphinothricin or basta (*e.g.,* bar gene); and glyphosate resistance (EPSPS gene)); and traits desirable for processing or process products such as high oil (*e.g.,* U.S. Patent No. 6,232,529); modified oils (*e.g.,* fatty acid desaturase genes (U.S. Patent No. 5,952,544; WO 94/11516)); modified starches (*e.g.,* ADPG pyrophosphorylases (AGPase), starch synthases (SS), starch branching enzymes (SBE) and starch debranching enzymes (SDBE)); and polymers or bioplastics (*e.g.,* U.S. patent No. 5.602,321; beta-ketothiolase, polyhydroxybutyrate synthase, and acetoacetyl-CoA reductase (Schubert et al. (1988) *J. Bacteriol.* 170:5837-5847) facilitate expression of polyhydroxyalkanoates (PHAs)), the disclosures of which are herein incorporated by reference. One could also combine the polynucleotides of the present invention with polynucleotides providing agronomic traits such as male sterility (*e.g.,* see U.S. Patent No. 5.583,210), stalk strength, flowering time, or transformation technology traits such as cell cycle regulation or gene targeting (*e.g.* WO 99/61619; WO 00/17364; WO 99/25821), the disclosures of which are herein incorporated by reference.

[0101] These stacked combinations can be created by any method including but not limited to cross breeding plants by any conventional or TopCross methodology, or genetic transformation. If the traits are stacked by genetically transforming the plants, the polynucleotide sequences of interest can be combined at any time and in any order. For example, a transgenic plant comprising one or more desired traits can be used as the target to introduce further traits by subsequent transformation. The traits can be introduced simultaneously in a co-transformation protocol with the polynucleotides of interest provided by any combination of transformation cassettes. For example, if two sequences will be introduced, the two sequences can be contained in separate transformation cassettes (trans) or contained on the same transformation cassette (cis). Expression of the sequences can be driven by the same promoter or by different promoters. In certain cases, it may be desirable to introduce a transformation cassette that will suppress the expression of the polynucleotide of interest. This may be combine with any combination of other suppression cassettes or overexpression cassettes to generate the desired combination of traits in the plant.

[0102] General texts which describe molecular biological techniques useful herein, including the use of vectors, promoters and many other relevant topics, include Berger and Kimmel, eds. (1987), *Guide to Molecular Cloning Techniques*, in *Methods Enzymol* 152: 673-684, Academic Press (hereinafter "Berger"); Sambrook and Ausubel. Examples of techniques sufficient to direct persons of skill through in vitro amplification methods, including the polymerase chain reaction (PCR), the ligase chain reaction (LCR), Qβ-replicase amplification

and other RNA polymerase mediated techniques (*e.g.,* NASBA), for the production of the homologous nucleic acids of the invention are found in Berger, Sambrook, and Ausubel, as well as U.S. Patent No. 4,683,202; Innis *et al.* eds. (1990) *PCR Protocols: A Guide to Methods and Applications*, Academic Press Inc. San Diego, CA; Arnheim & Levinson (1990) *Chemical & Engineering News* 36-47; *The Journal Of NIH Research* (1991) 3:81-94; Kwoh *et al.* (1989) *PNAS USA* 86:1173-1177; Guatelli *et al.* (1990) *PNAS USA* 87:1874-1878; Lomeli *et al.* (1989) *J Clin Chem* 35:1826-1831; Landegren *et al.*, (1988) *Science* 241:1077-1080; Van Brunt (1990) *Biotechnology* 8:291-294; Wu and Wallace, (1989) *Gene* 4:560-569; Barringer *et al.* (1990) *Gene* 89:117-122, and Sooknanan and Malek (1995) *Biotechnology* 13:563-564. Improved methods of cloning in vitro amplified nucleic acids are described in Wallace *et al.*, U.S. Pat. No. 5,426,039. Improved methods of amplifying large nucleic acids by PCR are summarized in Cheng *et al.* (1994) *Nature* 369:684-685 and the references therein, in which PCR amplicons of up to 40kb are generated. One of skill will appreciate that essentially any RNA can be converted into a double stranded DNA suitable for restriction digestion, PCR expansion and sequencing using reverse transcriptase and a polymerase. *See*, Ausubel, Sambrook and Berger, all *supra.*

[0103] The present invention also relates to host cells which are transformed with vectors of the invention, and the production of polypeptides of the invention by recombinant techniques. Host cells are genetically engineered (i.e., transduced, transformed or transfected) with the vectors of this invention, which may be, for example, a cloning vector or an expression vector. The vector may be, for example, in the form of a plasmid, a viral particle, a phage, etc. The engineered host cells can be cultured in conventional nutrient media modified as appropriate for activating promoters, selecting transformants, or amplifying the FD/FDD homologue gene. The culture conditions, such as temperature, pH, and the like, are those previously used with the host cell selected for expression, and will be apparent to those skilled in the art and in the references cited herein, including, *e.g.,* Payne *et al.* (1992) *Plant Cell and Tissue Culture in Liquid Systems* John Wiley & Sons, Inc. New York, NY (hereinafter "Payne") and the references cited therein.

Transgenic Plants

[0104] As noted herein, it is particularly desirable to transform plants with nucleic acids to reduce the level of fumonisins or fumonisin-derivatives in the plants. Reduction of such mycotoxins and/or their derivatives benefits the plants by making them resistant to mycotoxicosis, as well as making the plants safer for human and animal consumption. *See*, references cited *supra.*

[0105] In some embodiments, the present invention includes a cell comprising any nucleic acid (or vector) of the invention, which optionally expresses a polypeptide noted herein. In some embodiments, such cell expresses a polypeptide encoded by a nucleic acid. Typically, the polynucleotide and/or polypeptide are heterologous to the cell and are optionally operably linked to a regulatory sequence. Some such heterologous polynucleotides/polypeptides express/are exogenous polypeptides with fumonisin (and/or fumonisin-derivative) detoxification activity (*e.g.,* typically deamination). The fumonisin so detoxified is optionally a class B fumonisin, or is FB1. In some embodiments, the cells incorporating the nucleic acids and/or expressing the polypeptides of the invention are plant cells or fungal cells, or are bacterial cells. Transgenic plants, transgenic plant cells, and transgenic plant explants incorporating the nucleic acids of the invention are also features of the invention. In some embodiments, the transgenic plants, transgenic plant cells or transgenic plant explants express an exogenous polypeptide with fumonisin detoxification and/or fumonisin-derivative detoxification activity encoded by a nucleic acid of the invention. A seed produced by such transgenic plant is also a feature of the invention. In such instances, one or more parental codon of the nucleic acid can be substituted with a synonymous codon that is preferentially used by the translation machinery of a plant cell. Alternatively, the cell can be a microorganism cell, such as a bacteria, a fungus, or a yeast cell. In some embodiments, the transgenic plant into which any nucleic acid and/or polypeptide of the invention exists may be selected from the following: *Fragaria, Lotus, Medicago, Onobrychis, Trifolium, Trigonella, Vigna, Citrus, Linum, Geranium, Manihot, Daucus, Arabidopsis, Brassica, Raphanus, Sinapis, Atropa, Capsicum, Datura, Hyoscyamus, Lycopersicon, Nicotiana, Solanum, Petunia, Digitalis, Majorana, Cichorium, Helianthus, Lactuca, Bromus, Asparagus, Antirrhinum, Heterocallis, Nemesia, Pelargonium, Panicum, Pennisetum, Ranunculus, Senecio, Salpiglossis, Cucumis, Browaalia, Lolium, Malus, Apium, Gossypium, Vicia, Lathyrus, Lupinus, Pachyrhizus, Wisteria, Stizolobium, Agrostis, Phleum, Dactylis, Sorgum, Setaria, Zea, Oryza, Triticum, Secale, Avena, Hordeum, Saccharum, Poa, Festuca, Stenotaphrum, Cynodon, Coix, Olyreae, Phareae, Glycine, Pisum, Cicer, Phaseolus, Lens, Arachis,* corn, rice, cotton, soybean, sorghum, wheat, oat, barley, millet, sunflower, rapeseed, canola, pea, bean, lentil, peanut, yam, bean, cowpea, velvet bean, clover, alfalfa, lupine, vetch, lotus, sweet clover, wisteria, sweetpea, and a nut plant. It should be noted again, that as used herein, the term 'corn' is to be understood to refer to 'maize.'

[0106] Methods for producing transgenic organisms (*e.g.,* plants, fungi, bacteria, etc.) comprising a nucleic acid of the invention expressing a polypeptide at an effective level to deaminate fumonisin are also feature of the invention (*e.g.,* wherein the polypeptide expressed is a fumonisin amine oxidase). Additionally, other features of the invention

comprise methods of producing a transgenic plant or plant cell through: a) transforming a plant or plant cell with any polynucleotide of the invention (*e.g.*, any of SEQ ID NOs:1-25, SEQ ID NOs:67-69, and SEQ ID NOs:84, 86, 86, 99, 92, 94, 96, 98, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, 120, 122, 124, and 126 and fragments/modifications/complements/etc. of such) and b) optionally regenerating a transgenic plant from the transformed plant cell. A method for selecting a plant or cell containing a nucleic acid construct is also a feature of the invention wherein such method comprises: a) providing a transgenic plant or cell containing a nucleic acid construct comprising any nucleic acid of the invention, and b) growing the plant or cell in the presence of, *e.g.*, fumonisin under conditions where a polypeptide is expressed at an effective level whereby the transgenic plant or cell grows at a rate that is discernibly greater than the plant or cell would grow if it did not contain the nucleic acid construct. Also, a transgenic plant or explant that expresses any polypeptide of the invention (or fragment thereof which optionally encodes an active enzyme with FD/FDD activity) is a feature of the invention, as is such plant or explant that further expresses a polypeptide selected from: a polypeptide having fumonisin modification activity, a polypeptide having chitinase activity, a polypeptide having antifungal activity, a polypeptide having mycotoxin detoxification activity, a polypeptide having herbicidal activity, a polypeptide having pesticidal activity, and a polypeptide having nematicidal activity.

[0107] Therefore, transgenic plants, or plant cells, incorporating the FD/FDD nucleic acids, and/or expressing the corresponding polypeptides of the invention are a feature of the invention. The transformation of plant cells and protoplasts can be carried out in essentially any of the various ways known to those skilled in the art of plant molecular biology, including, but not limited to, the methods described herein. *See*, in general, Wu and Grossman, eds. (1987) *Methods Enzymol*, Vol. 153 (*Recombinant DNA Part D*), Academic Press, incorporated herein by reference. As used herein, the term "transformation" means alteration of the genotype of a host plant by the introduction of a nucleic acid sequence, *e.g.*, a "heterologous" or "foreign" nucleic acid sequence. The heterologous nucleic acid sequence need not necessarily originate from a different source but it will, at some point, have been external to the cell into which it is introduced.

[0108] In addition to Berger, Ausubel, Payne and Sambrook, useful general references for plant cell cloning, culture and regeneration include Jones (ed.) (1995) *Plant Gene Transfer and Expression Protocols-- Methods in Molecular Biology*, Vol. 49 Humana Press Towata, NJ (hereinafter "Jones"); and Gamborg and Phillips (eds.) (1995) *Plant Cell, Tissue and Organ Culture; Fundamental Methods* Springer Lab Manual, Springer-Verlag

(Berlin Heidelberg New York) (hereinafter "Gamborg"). A variety of cell culture media are described in Atlas and Parks (eds.) *The Handbook of Microbiological Media* (1993) CRC Press, Boca Raton, FL (hereinafter "Atlas"). Additional information for plant cell culture is found in available commercial literature such as the *Life Science Research Cell Culture Catalogue* (1998) from Sigma-Aldrich, Inc (St Louis, MO) (hereinafter "Sigma-LSRCCC") and, *e.g.,* the *Plant Culture Catalogue* and supplement (1997) also from Sigma-Aldrich, Inc (St Louis, MO) (hereinafter "Sigma-PCCS"). Additional details regarding plant cell culture are found in Croy, (ed.) (1993) *Plant Molecular Biology* Bios Scientific Publishers, Oxford, U.K. (hereinafter "Croy").

[0109] In an embodiment of this invention, recombinant vectors including one or more of the FD/FDD nucleic acids, *e.g.,* selected from SEQ ID NOs:1-25, SEQ ID NOs:67-69, and SEQ ID NOs:84, 86, 86, 99, 92, 94, 96, 98, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, 120, 122, 124, and 126, suitable for the transformation of plant cells are prepared. A DNA sequence encoding the desired FD/FDD protein, *e.g.,* selected from among SEQ ID NOs:26-50, SEQ ID NOs:70-72, and SEQ ID NOs:85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107, 109, 111, 113, 115, 117, 119, 121, 123, 125, and 127, is conveniently used to construct a recombinant expression cassette which can be introduced into a desired plant. In the context of the present invention, an expression cassette will typically comprise a selected FD/FDD nucleic acid sequence operably linked to a promoter sequence and other transcriptional and translational initiation regulatory sequences which are sufficient to direct the transcription of the FD/FDD sequence in the intended tissues (*e.g.,* entire plant, leaves, roots, etc.) of the transformed plant.

[0110] For example, a strongly or weakly constitutive plant promoter that directs expression of an FD/FDD nucleic acid in all tissues of a plant can be favorably employed. Such promoters are active under most environmental conditions and states of development or cell differentiation. Examples of constitutive promoters include the 1′- or 2′- promoter of *Agrobacterium tumefaciens*, and other transcription initiation regions from various plant genes known to those of skill. When overexpression of an FD/FDD nucleic acid of the invention is detrimental to the plant, one of skill will recognize that weak constitutive promoters can be used for low-levels of expression. In those cases where high levels of expression are not harmful to the plant, a strong promoter, *e.g.,* a t-RNA, or other pol III promoter, or a strong pol II promoter, (*e.g.,* the cauliflower mosaic virus promoter, CaMV, 35S promoter) can be used.

[0111] Alternatively, a plant promoter can be under environmental control. Such promoters are referred to as "inducible" promoters. Examples of environmental conditions that may alter transcription by inducible promoters include pathogen attack, anaerobic conditions, or the presence of light. In some cases, it is desirable to use promoters that are "tissue-specific" and/or are under developmental control such that the FD/FDD gene is expressed only in certain tissues or stages of development, *e.g.,* leaves, roots, shoots, etc. Endogenous promoters of genes related to herbicide tolerance and related phenotypes are particularly useful for driving expression of FD/FDD nucleic acids, *e.g.,* P450 monooxygenases, glutathione-S-transferases, homoglutathione-S-transferases, glyphosate oxidases and 5-enolpyruvyl-shikimate-2-phosphate synthases.

[0112] Tissue specific promoters can also be used to direct expression of heterologous structural genes, including the FD/FDD nucleic acids described herein. Thus the promoters can be used in recombinant expression cassettes to drive expression of any gene whose expression is desirable in the transgenic plants of the invention, *e.g.,* FD/FDD and/or other genes conferring fumonisin neutralizing capability, or genes which influence other useful characteristics, *e.g.,* heterosis.

[0113] In general, the particular promoter used in the expression cassette in plants depends on the intended application. Any of a number of promoters which direct transcription in plant cells can be suitable. The promoter can be either constitutive or inducible. In addition to the promoters noted above, promoters of bacterial origin which operate in plants include the octopine synthase promoter, the nopaline synthase promoter and other promoters derived from Ti plasmids. *See, e.g.,* Herrera-Estrella *et al.* (1983) *Nature* 303:209. Viral promoters include the 35S and 19S RNA promoters of CaMV. *See, e.g.,* Odell *et al.*, (1985) *Nature* 313:810. Other plant promoters include the ribulose-1,3-bisphosphate carboxylase small subunit promoter and the phaseolin promoter. The promoter sequence from the E8 gene (*see*, Deikman and Fischer (1988) *EMBO J* 7:3315) and other genes are also favorably used. Alternatively, novel promoters with useful characteristics can be identified from any viral, bacterial, or plant source by methods, including sequence analysis, enhancer or promoter trapping, and the like, known in the art.

[0114] In preparing expression vectors containing any of the FD/FDD encoding nucleic acids of the invention, sequences other than the promoter and the recursively recombined gene are also favorably used. If proper polypeptide expression is desired, a polyadenylation region can be derived from the natural gene, from a variety of other plant genes, or from T-DNA. Signal/localization peptides, which, *e.g.,* facilitate translocation of

the expressed polypeptide to internal organelles (*e.g.*, chloroplasts) or extracellular secretion, can also be employed.

[0115] The vector comprising the FD/FDD nucleic acid also typically includes a marker gene which confers a selectable phenotype on plant cells. For example, the marker may encode biocide tolerance, particularly antibiotic tolerance, such as tolerance to kanamycin, G418, bleomycin, hygromycin, or herbicide tolerance, such as tolerance to chlorosulfuron, or phosphinothricin. Reporter genes, which are used to monitor gene expression and protein localization via visualizable reaction products (*e.g.*, beta-glucuronidase, beta-galactosidase, and chloramphenicol acetyltransferase) or by direct visualization of the gene product itself (*e.g.*, green fluorescent protein, GFP, *see*, *e.g.*, Sheen *et al.* (1995) *Plant J* 8:777) can be used for, *e.g.*, monitoring transient gene expression in plant cells. Transient expression systems can be employed in plant cells, for example, in screening plant cell cultures for herbicide tolerance activities or, as in the present case, for FD/FDD activity.

Plant transformation

Protoplasts

[0116] Numerous protocols for establishment of transformable protoplasts from a variety of plant types and subsequent transformation of the cultured protoplasts are available in the art and are incorporated herein by reference. For examples, *see*, Hashimoto *et al.* (1990) *Plant Physiol* 93:857; Fowke and Constabel (eds.) (1994) *Plant Protoplasts*; Saunders *et al.* (1993) *Applications of Plant In Vitro Technology Symposium*, UPM 16-18; and Lyznik *et al.* (1991) *BioTechniques* 10:295, each of which is incorporated herein by reference.

Chloroplasts

[0117] Chloroplasts are a site of action for many activities, and, in some instances, the FD/FDD sequences may be fused to chloroplast transit sequence peptides to facilitate translocation of the gene products into the chloroplasts. In these cases, it can be advantageous to transform the FD/FDD nucleic acids into chloroplasts of the plant host cells. Numerous methods are available in the art to accomplish chloroplast transformation and expression (*see*, *e.g.*, Daniell *et al.* (1998) *Nat Biotechnol* 16:346; O'Neill *et al.* (1993) *Plant J* 3:729; Maliga (1993) *TIBTECH* 11:1). The expression construct comprises a transcriptional regulatory sequence, functional in plants, operably linked to a polynucleotide encoding the FD/FDD polypeptide. Expression cassettes that are designed to function in chloroplasts (such as an expression cassette including an FD/FDD nucleic acid) include the sequences necessary to ensure expression in chloroplasts. Typically, the coding sequence is flanked by two regions

of homology to the chloroplastid genome to effect a homologous recombination with the chloroplast genome; often a selectable marker gene is also present within the flanking plastid DNA sequences to facilitate selection of genetically stable transformed chloroplasts in the resultant transplastonic plant cells (*see, e.g.,* Maliga (1993) and Daniell (1998), *supra,* and references cited therein).

General plant transformation methods

[0118] DNA constructs containing any of the FD/FDD nucleic acids of the invention can be introduced into the genome of the desired plant host by a variety of conventional techniques. Techniques for transforming a wide variety of higher plant species are well known and described in the technical and scientific literature. *See, e.g.,* Payne, Gamborg, Croy, Jones, etc. all *supra,* as well as, *e.g.,* Weising *et al.* (1988) *Ann Rev Genet* 22:421.

[0119] For example, DNAs can be introduced directly into the genomic DNA of a plant cell using techniques such as electroporation or micro-injection of plant cell protoplasts, or the DNA constructs can be introduced directly to plant tissue using ballistic methods, such as DNA particle bombardment. Alternatively, the DNA constructs can be combined with suitable T-DNA flanking regions and introduced into a conventional *Agrobacterium tumefaciens* host vector. The virulence functions of the Agrobacterium host will direct the insertion of the construct and adjacent marker into the plant cell DNA when the plant cell is infected by the bacteria.

[0120] Micro-injection techniques are known in the art and well described in the scientific and patent literature. The introduction of DNA constructs using polyethylene glycol precipitation is described in Paszkowski et al (1984) *EMBO J* 3:2717. Electroporation techniques are described in Fromm *et al.* (1985) *PNAS USA* 82:5824. Ballistic transformation techniques are described in Klein *et al.* (1987) *Nature* 327:70; and Weeks *et al. Plant Physiol* 102:1077.

[0121] In some embodiments, Agrobacterium mediated transformation techniques are used to transfer the FD/FDD sequences of the invention into plants. Agrobacterium-mediated transformation is widely used for the transformation of dicots, however, certain monocots can also be transformed by Agrobacterium. For example, Agrobacterium transformation of rice is described by Hiei *et al.* (1994) *Plant J* 6:271; US Patent No. 5,187,073; US Patent No. 5,591,616; Li *et al.* (1991) *Sci China* 34:54; and Raineri *et al.* (1990) *Bio/Technology* 8:33. Transformed maize, barley, triticale and asparagus by Agrobacterium mediated transformation have also been described (Xu *et al.* (1990) *Chin J Bot* 2:81).

[0122] Agrobacterium mediated transformation techniques take advantage of the ability of the tumor-inducing (Ti) plasmid of *A. tumefaciens* to integrate into a plant cell genome and to co-transfer a nucleic acid of interest (*e.g.*, any nucleic acid of the present invention encoding FD/FDD capability, or a fragment of such nucleic acid) into a plant cell. Typically, an expression vector is produced wherein the nucleic acid of interest, again, such as an FD/FDD nucleic acid of the invention, is ligated into an autonomously replicating plasmid which also contains T-DNA sequences. T-DNA sequences typically flank the expression cassette nucleic acid of interest and comprise the integration sequences of the plasmid. In addition to the expression cassette, T-DNA also typically includes a marker sequence, *e.g.*, antibiotic resistance genes. The plasmid with the T-DNA and the expression cassette are then transfected into Agrobacterium cells. Typically, for effective transformation of plant cells, the *A. tumefaciens* bacterium also possesses the necessary *vir* regions on a plasmid, or integrated into its chromosome. For a discussion of Agrobacterium mediated transformation, *see*, Firoozabady and Kuehnle, (1995) in Gamborg (*supra*).

Regeneration of Transgenic Plants

[0123] Transformed plant cells which are derived by plant transformation techniques, including those discussed above, can be cultured to regenerate a whole plant which possesses the transformed genotype (*e.g.*, an FD/FDD nucleic acid or fragment thereof), and thus the desired phenotype, such as, *e.g.*, the capability to detoxify, degrade or neutralize fumonisins or fumonisin-derivatives. Such regeneration techniques rely on manipulation of certain phytohormones in a tissue culture growth medium, typically relying on a biocide and/or herbicide marker which has been introduced together with the desired nucleotide sequences. Alternatively, selection for the capability to detoxify, degrade, or neutralize fumonisins or fumonisin-derivatives conferred by the FD/FDD nucleic acids of the invention can be performed. Plant regeneration from cultured protoplasts is described in Evans *et al.* (1983) *Protoplasts Isolation and Culture, Handbook of Plant Cell Culture*, pp. 124-176, Macmillan Publishing Company, New York; and Binding (1985) *Regeneration of Plants, Plant Protoplasts* pp. 21-73, CRC Press, Boca Raton, FL. Regeneration can also be obtained from plant callus, explants, organs, or parts thereof. Such regeneration techniques are described generally in Klee et al. (1987) *Ann Rev Plant Phys* 38:467. *See also, e.g.,* Payne and Gamborg.

[0124] After transformation with Agrobacterium, the explants are transferred to selection medium. One of skill will realize that the selection medium depends on the selectable marker that was co-transfected into the explants. After a suitable length of time, transformants will begin to form shoots. After the shoots are, *e.g.*, about 1-2 cm in length, the

shoots should be transferred to a suitable root and shoot medium. Selection pressure should be maintained in the root and shoot medium.

[0125] Typically, the transformants will develop roots in, *e.g.*, about 1-2 weeks and form plantlets. After the plantlets are, *e.g.*, about 3-5 cm in height, they are placed in, e.g., sterile soil in fiber pots. Those of skill in the art will realize that different acclimation procedures are used to obtain transformed plants of different species. For example, after developing a root and shoot, cuttings, as well as somatic embryos of transformed plants, are transferred to medium for establishment of plantlets. For a description of selection and regeneration of transformed plants, *see*, *e.g.*, Dodds and Roberts (1995) *Experiments in Plant Tissue Culture*, 3rd Ed., Cambridge University Press.

[0126] The transgenic plants of this invention can be characterized either genotypically or phenotypically to determine the presence of the FD/FDD nucleic acids of the invention. Genotypic analysis can be performed by any of a number of well-known techniques, including PCR amplification of genomic DNA and hybridization of genomic DNA with specific labeled probes. Phenotypic analysis includes, *e.g.*, survival of plants in the presence of a selected fumonisin or fumonisin-derivative. Other types of selections for fumonisin resistance or fumonisin-derivative resistance are discussed *infra*.

[0127] Essentially any plant can be transformed with the FD/FDD nucleic acids of the invention. Suitable plants for the transformation and expression of the novel FD/FDD nucleic acids of this invention include agronomically and horticulturally important species. Such species include, but are not restricted to members of the families: Graminae (including corn, rye, triticale, barley, millet, rice, wheat, oat, etc.); Leguminosae (including pea, bean, lentil, peanut, yam bean, cowpea, velvet bean, soybean, clover, alfalfa, lupine, vetch, lotus, sweet clover, wisteria, and sweetpea); Compositae (the largest family of vascular plants, including at least 1,000 genera, including important commercial crops such as sunflower) and Rosaciae (including raspberry, apricot, almond, peach, rose, etc.), as well as nut plants (including, walnut, pecan, hazelnut, etc.), and forest trees (including *Pinus, Quercus, Pseudotsuga, Sequoia, Populus*, etc.)

[0128] Additionally, preferred targets for modification by the FD/FDD nucleic acids of the invention, as well as those specified above, include plants from the genera: *Agrostis, Allium, Antirrhinum, Apium, Arachis, Asparagus, Atropa, Avena* (*e.g.*, oat), *Bambusa, Brassica, Bromus, Browaalia, Camellia, Cannabis, Capsicum, Cicer, Chenopodium, Chichorium, Citrus, Coffea, Coix, Cucumis, Curcubita, Cynodon, Dactylis, Datura, Daucus, Digitalis, Dioscorea, Elaeis, Eleusine, Festuca, Fragaria, Geranium, Glycine, Helianthus,*

Heterocallis, Hevea, Hordeum (*e.g.*, barley), *Hyoscyamus, Ipomoea, Lactuca, Lens, Lilium, Linum, Lolium, Lotus, Lycopersicon, Majorana, Malus, Mangifera, Manihot, Medicago, Nemesia, Nicotiana, Onobrychis, Oryza* (*e.g.*, rice), *Panicum, Pelargonium, Pennisetum* (*e.g.*, millet), *Petunia, Pisum, Phaseolus, Phleum, Poa, Prunus, Ranunculus, Raphanus, Ribes, Ricinus, Rubus, Saccharum, Salpiglossis, Secale* (*e.g.*, rye), *Senecio, Setaria, Sinapis, Solanum, Sorghum, Stenotaphrum, Theobroma, Trifolium, Trigonella, Triticum* (*e.g.*, wheat), *Vicia, Vigna, Vitis, Zea* (*e.g.*, corn), and the *Olyreae*, the *Pharoideae* and many others. As noted, plants in the family *Graminae* are a particularly preferred target plants for the methods of the invention.

[0129] Common crop plants which are targets of the present invention include corn, rice, triticale, rye, cotton, soybean, sorghum, wheat, oat, barley, millet, sunflower, canola, pea, bean, lentil, peanut, yam bean, cowpea, velvet bean, clover, alfalfa, lupine, vetch, lotus, sweet clover, wisteria, sweetpea and nut plants (*e.g.*, walnut, pecan, etc).

Non-Plant Expression Vectors/Systems

[0130] The FD/FDD homologue proteins of the invention can also be produced in non-plant cells such as animals, yeast, fungi, bacteria and the like. In addition to Sambrook, Berger, Gamborg, Atlas and Ausubel, details regarding cell culture can be found in; *e.g.*, Freshney (1994) *Culture of Animal Cells, a Manual of Basic Technique*, third edition, Wiley-Liss, New York (hereinafter "Freshney").

[0131] The polynucleotides of the present invention and fragments thereof, which encode the FD/FDD molecules, may be included in any one of a variety of expression vectors for expressing a polypeptide. Such vectors include chromosomal, non-chromosomal and synthetic DNA sequences, *e.g.*, derivatives of SV40, bacterial plasmids, phage DNA, baculovirus, yeast plasmids, vectors derived from combinations of plasmids and phage DNA, viral DNA such as vaccinia, adenovirus, fowl pox virus, pseudorabies, adeno-associated virus, retroviruses and many others. Any vector that transduces genetic material into a cell, and, if replication is desired, which is replicable and viable in the relevant host can be used.

[0132] The nucleic acid sequence in the expression vector is operatively linked to an appropriate transcription control sequence (promoter) to direct mRNA synthesis. Examples of such promoters include: CaMV, LTR or SV40 promoter, *E. coli* lac or trp promoter, phage lambda P_L promoter, and other promoters known to control expression of genes in prokaryotic or eukaryotic cells or their viruses. The expression vector also contains a ribosome binding site for translation initiation, and a transcription terminator. The vector optionally includes

appropriate sequences for amplifying expression, *e.g.*, an enhancer. In addition, the expression vectors optionally comprise one or more selectable marker genes to provide a phenotypic trait for selection of transformed host cells, such as dihydrofolate reductase or neomycin resistance for eukaryotic cell culture, or such as tetracycline or ampicillin resistance in *E. coli*.

[0133] The vector containing the appropriate DNA sequence encoding the FD/FDD polypeptide, as well as an appropriate promoter or control sequence, may be employed to transform an appropriate host to permit the host to express the protein. Examples of appropriate expression hosts include: bacterial cells, such as *E. coli*, *Streptomyces*, and *Salmonella typhimurium*; fungal cells, such as *Saccharomyces cerevisiae, Pichia pastoris,* and *Neurospora crassa*; insect cells such as *Drosophila* and *Spodoptera frugiperda*; mammalian cells such as CHO, COS, BHK, HEK 293 or Bowes melanoma; plant cells or explants, etc. It is understood that not all cells or cell lines need to be capable of producing fully functional FD/FDD homologues; for example, antigenic fragments of an FD/FDD homologue may be produced in a bacterial or other expression system. The invention is not limited by the host cells employed.

[0134] In bacterial systems, a number of expression vectors may be selected depending upon the use intended for the FD/FDD homologue. For example, when large quantities of FD/FDD homologue or fragments thereof are needed for the induction of antibodies, vectors which direct high level expression of fusion proteins that are readily purified may be desirable. Such vectors include, but are not limited to, multifunctional *E. coli* cloning and expression vectors such as BLUESCRIPT (Stratagene), in which the FD/FDD homologue coding sequence may be ligated into the vector in-frame with sequences for the amino-terminal Met and the subsequent 7 residues of beta-galactosidase so that a hybrid protein is produced; pIN vectors (Van Heeke & Schuster (1989) *J Biol Chem* 264:5503-5509); pET vectors (Novagen, Madison WI); and the like.

[0135] Similarly, in the yeast *Saccharomyces cerevisiae*, a number of vectors containing constitutive or inducible promoters such as alpha factor, alcohol oxidase and PGH may be used for production of the FD/FDD homologue proteins of the invention. For reviews, see Ausubel, Berger, and Grant *et al.* (1987; *Methods Enzymol* 153:516-544).

[0136] In mammalian host cells, a number of expression systems, such as viral-based systems, may be utilized. In cases where an adenovirus is used as an expression vector, a coding sequence is optionally ligated into an adenovirus transcription/translation complex consisting of the late promoter and tripartite leader sequence. Insertion in a nonessential E1

or E3 region of the viral genome will result in a viable virus capable of expressing FD/FDD homologues in infected host cells (Logan and Shenk (1984) *PNAS USA* 81:3655-3659). In addition, transcription enhancers, such as the rous sarcoma virus (RSV) enhancer, may be used to increase expression in mammalian host cells.

[0137] Similarly, in plant cells, expression can be driven from a transgene integrated into a plant chromosome, or cytoplasmically from an episomal or viral nucleic acid. In the case of stably integrated transgenes, it is often desirable to provide sequences capable of driving constitutive or inducible expression of the genes, *e.g.*, the FD/FDD homologue sequences of the invention. Numerous plant derived regulatory sequences have been described, including sequences which direct expression in a tissue specific manner, *e.g.*, TobRB7, patatin B33, GRP gene promoters, the rbcS-3A promoter, and the like. Alternatively, high level expression can be achieved by transiently expressing exogenous sequences of a plant viral vector, *e.g.*, CaMV, TMV, BMV, etc.

Additional Expression Elements

[0138] Specific initiation signals can aid in efficient translation of an FD/FDD homologue coding sequence and fragments thereof. These signals can include, *e.g.*, the ATG initiation codon and adjacent sequences. In cases where an FD/FDD homologue coding sequence and its initiation codon and upstream sequences are inserted into the appropriate expression vector, no additional translational control signals may be needed. However, in cases where only coding sequence (*e.g.*, a mature protein coding sequence), or a portion thereof, is inserted, exogenous transcriptional control signals including the ATG initiation codon must be provided. Furthermore, the initiation codon must be in the correct reading frame to ensure transcription of the entire insert. Exogenous transcriptional elements and initiation codons can be of various origins, both natural and synthetic. The efficiency of expression may be enhanced by the inclusion of enhancers appropriate to the cell system in use (*see*, *e.g.*, Scharf D. *et al.* (1994) *Results Probl Cell Differ* 20:125-62; Bittner *et al.* (1987) *Methods Enzymol* 153:516-544).

Secretion/Localization Sequences

[0139] Polynucleotides of the invention encoding the FD/FDD homologues and fragments thereof can also be fused, for example, in-frame to a nucleic acid encoding a secretion/localization sequence, to target polypeptide expression to a desired cellular compartment, membrane, or organelle, or to direct polypeptide secretion to the periplasmic space or into the cell culture media. Such sequences are known to those of skill, and include secretion leader peptides, organelle targeting sequences (*e.g.*, nuclear localization sequences,

ER retention signals, mitochondrial transit sequences, chloroplast transit sequences), membrane localization/anchor sequences (*e.g.*, stop transfer sequences, GPI anchor sequences), and the like. Signal peptides for localization in the apoplastic space (i.e., extracellular space) are also optionally used in combination with the FD/FDD homologues of the current invention. *See, e.g.*, the PR1b signal sequence as described in Lind *et al.* (1992), *Plant Mol Biol* 18:47-53, PR-1a, b and c signals described in Pfitzner *et al.* (1987), *Nucl Acids Res* 15:4449-4465 or the barley alpha amylase (BAA) secretion sequence (Rahmatullah *et al.* (1989) *Plant Mol Biol* 12:119). Similarly, localization to peroxisomal space, *e.g.*, peroxisomal targeting, is optionally accomplished through use of, *e.g.*, the signal described by Keller *et al.* (1991) *J Cell Biol*, 114, p. 893.-904.

Expression Hosts

[0140] In a further embodiment, the present invention relates to host cells containing the above-described constructs. The host cell can be a eukaryotic cell, such as a mammalian cell, a yeast cell, or a plant cell, or the host cell can be a prokaryotic cell, such as a bacterial cell. Introduction of the construct into the host cell can be effected by calcium phosphate transfection, DEAE-Dextran mediated transfection, electroporation, or other common techniques (*see, e.g.*, Davis *et al.* (1986) *Basic Methods in Molecular Biology*, Elsevier, New York.).

[0141] A host cell strain is optionally chosen for its ability to modulate the expression of the inserted sequences or to process the expressed protein (*e.g.*, a fumonisin detoxification enzyme) in the desired fashion. Such modifications of the protein include, but are not limited to, acetylation, carboxylation, glycosylation, phosphorylation, lipidation and acylation. Post-translational processing which cleaves a "pre" or a "prepro" form of the protein may also be important for correct insertion, folding and/or function. Different host cells such as *E. coli, Bacillus* sp., yeast or mammalian cells such as CHO, HeLa, BHK, MDCK, 293, WI38, etc. have specific cellular machinery and characteristic mechanisms for such post-translational activities and may be chosen to ensure the correct modification and processing of the introduced, foreign protein.

[0142] For long-term, high-yield production of recombinant proteins, stable expression can be used. For example, plant cells, explants or tissues (*e.g.*, shoots or leafdiscs) which stably express an FD/FDD polypeptide of the invention are transduced using expression vectors which contain viral origins of replication or endogenous expression elements and a selectable marker gene. Following the introduction of the vector, cells may be allowed to grow for a period of time appropriate for the cell type (*e.g.*, 1 or more hours for

bacterial cells, 1-4 days for plant cells, or 2-4 weeks for some plant explants) before they are switched to a selective medium. The purpose of the selectable marker is to confer resistance to selection, and its presence allows growth and recovery of cells that successfully express the introduced sequences. For example, resistant clumps of stably transformed cells can be proliferated using tissue culture techniques appropriate to the cell type.

[0143] Host cells transformed with a nucleotide sequence encoding an FD/FDD polypeptide capable of detoxifying, degrading, or neutralizing a fumonisin or a fumonisin-derivative are optionally cultured under conditions suitable for the expression and recovery of the encoded protein from cell culture. The protein or fragment thereof produced by a recombinant cell may be secreted, membrane-bound, or contained intracellularly, depending on the sequence and/or the vector used. As will be understood by those of skill in the art, expression vectors containing polynucleotides encoding mature FD/FDD homologues of the invention can be designed with signal sequences which direct secretion of the mature polypeptides through a prokaryotic or eukaryotic cell membrane.

Additional Polypeptide and Polynucleotide Sequences

[0144] The FD/FDD polypeptide encoding polynucleotides of the present invention may also comprise a coding sequence or fragment thereof fused in-frame to a marker sequence which, *e.g.*, facilitates purification of the encoded polypeptide. Such purification facilitating domains include, but are not limited to, metal chelating peptides such as histidine-tryptophan modules that allow purification on immobilized metals, a sequence which binds glutathione (*e.g.*, GST), a hemagglutinin (HA) tag (corresponding to an epitope derived from the influenza hemagglutinin protein; Wilson, I., *et al.* (1984) *Cell* 37:767), maltose binding protein sequences, the FLAG epitope utilized in the FLAGS extension/affinity purification system (Immunex Corp, Seattle, WA), and the like. The inclusion of a protease-cleavable polypeptide linker sequence between the purification domain and the FD/FDD homologue sequence is useful to facilitate purification.

[0145] For example, one expression vector possible to use in the compositions and methods described herein provides for expression of a fusion protein comprising a polypeptide of the invention fused to a polyhistidine region separated by an enterokinase cleavage site. The histidine residues facilitate purification on IMIAC (immobilized metal ion affinity chromatography, as described in Porath *et al.* (1992) *Protein Expression Purif* 3:263-281, while the enterokinase cleavage site provides a method for separating the FD/FDD homologue polypeptide from the fusion protein. pGEX vectors (Amersham Pharmacia Biotech) are optionally used to express foreign polypeptides as fusion proteins with

glutathione S-transferase (GST). Other expression systems, such as, *e.g.*, pPICz vectors (Invitrogen) that allow for expression in *Pichia* are also optionally used. In general, such fusion proteins are soluble and can easily be purified from lysed cells by adsorption to ligand-agarose beads (*e.g.*, glutathione-agarose in the case of GST-fusions) followed by elution in the presence of free ligand.

Polypeptide Production and Recovery

[0146] Following transformation of a suitable host strain and growth of the host strain to an appropriate cell density, the selected promoter is induced by appropriate means (*e.g.*, temperature shift or chemical induction) and cells are cultured for an additional period. Cells are typically harvested by centrifugation, disrupted by physical or chemical means, and the resulting crude extract retained for further purification. Eukaryotic or microbial cells employed in expression of the FD/FDD proteins can be disrupted by any convenient method, including freeze-thaw cycling, sonication, mechanical disruption, or use of cell lysing agents, or other methods, which are well know to those skilled in the art.

[0147] As noted, many references are available for the culture and production of many cells, including cells of bacterial, plant, animal (especially mammalian) and archebacterial origin. *See e.g.*, Sambrook, Ausubel, Freshney and Berger, as well as Doyle and Griffiths (1997) *Mammalian Cell Culture: Essential Techniques* John Wiley and Sons, NY; Humason (1979) *Animal Tissue Techniques*, fourth edition W.H. Freeman and Company; and Ricciardelli, *et al.*, (1989) In vitro *Cell Dev Biol* 25:1016-1024. For plant cell culture and regeneration see, Payne, Gamborg, and Croy. Cell culture media in general are set forth in Atlas. Additional information for cell culture is found in available commercial literature such as Sigma-LSRCCC and Sigma-PCCS.

[0148] Polypeptides of the invention can be recovered and purified from recombinant cell cultures by any of a number of methods well known in the art, including ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography (*e.g.*, using any of the tagging systems noted herein), hydroxylapatite chromatography, and lectin chromatography. Protein refolding steps can be used, as desired, in completing configuration of the mature FD/FDD proteins or fragments thereof. Finally, high performance liquid chromatography (HPLC) can be employed in the final purification steps. In addition to the references noted *supra*, a variety of purification methods are well known in the art, including, *e.g.*, those set forth in Sandana (1997) *Bioseparation of Proteins*, Academic Press, Inc.; Bollag *et al.* (1996) *Protein Methods*,

2nd Edition, Wiley-Liss, NY; Walker (1996) *The Protein Protocols Handbook* Humana Press, NJ; Harris and Angal (1990) *Protein Purification Applications: A Practical Approach*, IRL Press at Oxford, Oxford, England; Scopes (1993) *Protein Purification: Principles and Practice*, 3rd Edition, Springer Verlag, NY; Janson and Ryden (1998) *Protein Purification: Principles, High Resolution Methods and Applications*, Second Edition Wiley-VCH, NY; and Walker (1998) *Protein Protocols on CD-ROM* Humana Press, NJ (hereinafter "Walker").

In vitro Expression Systems

[0149] Cell-free transcription/translation systems can also be employed to produce FD/FDD polypeptides or fragments thereof using DNAs or RNAs of the present invention. Several such systems are commercially available. A general guide to in vitro transcription and translation protocols is found in Tymms (1995) *In vitro Transcription and Translation Protocols: Methods in Molecular Biology* Volume 37, Garland Publishing, NY.

Modified Amino Acids

[0150] Polypeptides of the invention may contain one or more modified amino acid. The presence of modified amino acids may be advantageous in, for example, (a) increasing polypeptide half-life, (b) reducing polypeptide antigenicity, or (c) increasing polypeptide storage stability. Amino acid(s) are modified, for example, co-translationally or post-translationally during recombinant production (*e.g.*, N-linked glycosylation at N-X-S/T motifs during expression in mammalian cells) or modified by synthetic means.

[0151] Non-limiting examples of a modified amino acid include a glycosylated amino acid, a sulfated amino acid, a prenlyated (*e.g.*, farnesylated, geranylgeranylated) amino acid, an acetylated amino acid, an acylated amino acid, a PEG-ylated amino acid, a biotinylated amino acid, a carboxylated amino acid, a phosphorylated amino acid, and the like. References adequate to guide one of skill in the modification of amino acids are replete throughout the literature. Example protocols are found in Walker.

In Vivo Uses

[0152] Polynucleotides or fragments thereof which encode an FD/FDD homologue polypeptide of the invention, or complements of the polynucleotides (*e.g.*, antisense or ribozyme molecules), are optionally administered to a cell to accomplish a useful process or to express a useful product. These *in vivo* applications, including gene therapy, include a multitude of techniques by which gene expression may be altered in cells. Such methods

include, for instance, the introduction of genes for expression of, *e.g.*, useful polypeptides, such as the FD/FDD homologues of the present invention or fragments thereof.

[0153] There are many applications involving *in vivo* use of FD/FDD polynucleotides of the invention. Non-limiting examples include the following scenarios. One example of *in vivo* use for the FD/FDD polynucleotides of the invention involves large scale production of FD/FDD enzyme for use as a fumonisin (or fumonisin-derivative) detoxification treatment for foodstuffs and/or crops (*e.g.*, as a treatment for cereals, grains, silages, etc.). Those skilled in the art can use the FD/FDD homologues of the invention to transform micro-organisms (*e.g.*, any of numerous species of bacteria and/or yeasts), *see, supra*, in order to create large scale production of the FD/FDD enzymes. The polypeptides thus produced can optionally be isolated and/or purified from the micro-organisms and applied during, *e.g.*, the processing, storage, and/or production of the foodstuff and/or crop involved (*e.g.*, maize (i.e., corn), etc.). Optionally and/or alternatively, the micro-organism expressing the FD/FDD polypeptides of the invention can be itself applied to the foodstuff and/or crop in question, (as, *e.g.*, a live organism and/or non-refined non-living preparation such as lyophilized preparation).

[0154] Additionally, the polypeptides produced from expression of the FD/FDD polynucleotides of the invention in large scale production can be used as components of systems that test for certain mycotoxin contamination of crops and/or foodstuffs. *See*, Example 1, for an example of one type of procedure which can be used to test for the contamination of foodstuffs and/or crops. Use of the polypeptides and/or polynucleotides of the invention in testing for fumonisin or fumonisin-derivative (or similar mycotoxins) contamination of foodstuffs and/or plants, etc. can also be incorporated into kits or pre-prepared formats for testing of foodstuffs. Such kits and uses are part of the invention

[0155] Another optional example of use of the FD/FDD molecules of the invention is the generation of transgenic plants expressing one or more FD/FDD polypeptide of the present invention. *See, supra.* Such transgenic plants are able to eliminate or ameliorate the levels of fumonisin contamination, thereby not only making the plants and/or their products safer for human and/or animal consumption, but also making the plants more resistant to pathogenic organisms that use such mycotoxins as a mode of entry to infect the plants. A possible additional benefit of fumonisin/fumonisin-derivative breakdown by the polypeptides of the invention is the production of a hydrogen peroxide by-product since hydrogen peroxide has anti-microbial activity, acts as a substrate for enzymes involved in cell wall strength, acts as a signal to activate plant defense genes (*e.g.*, those involved in salicylic acid build-up

which is involved in gene expression of pathogenesis related proteins, and might be involved in the production/expression of other plant defense compounds such as phytoalexins, etc.

[0156] An additional, but non-limiting, example of a use for the current invention is for the production of, *e.g.*, oxidized fumonisins and/or oxidized fumonisin-derivatives for use in, *e.g.*, research or the like.

Antisense Technology

[0157] In addition to expression of the FD/FDD nucleic acids of the invention as gene replacement nucleic acids, the nucleic acids are also useful for sense and anti-sense suppression of expression, *e.g.*, to down-regulate expression of an FD/FDD encoding nucleic acid of the invention, once, or when, expression of the nucleic acid is no longer desired in the cell. Similarly, the nucleic acids of the invention, or subsequences or anti-sense sequences thereof, can also be used to block expression of naturally occurring homologous nucleic acids. A variety of sense and anti-sense technologies are known in the art, *e.g.*, as set forth in Lichtenstein and Nellen (1997) *Antisense Technology: A Practical Approach* IRL Press at Oxford University, Oxford, England, and in Agrawal (1996) *Antisense Therapeutics* Humana Press, NJ, and the references cited therein. One optional non-limiting example of the use of antisense regulation of FD/FDD is for, *e.g.*, the timing of production of FD/FDD polypeptides to coincide with specific periods in the life-cycle of transgenic plants (*e.g.*, production of FD/FDD polypeptides only during fruiting or right before harvest, etc.).

Use as Probes

[0158] Also contemplated are uses of polynucleotides, also referred to herein as oligonucleotides, typically having at least 12 bases, sometimes at least 15, occasionally at least 20, 25, 30, 35, 40, 45, or 50 or more bases, which hybridize under highly stringent conditions to an FD/FDD homologue polynucleotide sequence described herein or a fragment thereof. The polynucleotides may be used as probes, primers, sense and antisense agents, and the like, according to methods as noted *supra*. One non-limiting example of the use of FD/FDD sequences of the invention as probes involves their use, *e.g.*, in the discovery or synthesis of new fumonisin or other mycotoxin degrading enzymes. The polynucleotide sequences of the invention, or fragments thereof, can be used to screen both naturally occurring and synthetically constructed groups of polypeptides for ones which are similar to the products of the invention and which therefore may show useful fumonisin degrading abilities.

SEQUENCE VARIATIONS

Silent Variations

[0159] It will be appreciated by those skilled in the art that due to the degeneracy of the genetic code, a multitude of nucleic acids sequences encoding FD/FDD homologue polypeptides of the invention may be produced, some which may bear minimal sequence homology to the nucleic acid sequences explicitly disclosed herein.

Table 1

Codon Table

Amino acids			Codon					
Alanine	Ala	A	GCA	GCC	GCG	GCU		
Cysteine	Cys	C	UGC	UGU				
Aspartic acid	Asp	D	GAC	GAU				
Glutamic acid	Glu	E	GAA	GAG				
Phenylalanine	Phe	F	UUC	UUU				
Glycine	Gly	G	GGA	GGC	GGG	GGU		
Histidine	His	H	CAC	CAU				
Isoleucine	Ile	I	AUA	AUC	AUU			
Lysine	Lys	K	AAA	AAG				
Leucine	Leu	L	UUA	UUG	CUA	CUC	CUG	CUU
Methionine	Met	M	AUG					
Asparagine	Asn	N	AAC	AAU				
Proline	Pro	P	CCA	CCC	CCG	CCU		
Glutamine	Gln	Q	CAA	CAG				
Arginine	Arg	R	AGA	AGG	CGA	CGC	CGG	CGU
Serine	Ser	S	AGC	AGU	UCA	UCC	UCG	UCU
Threonine	Thr	T	ACA	ACC	ACG	ACU		
Valine	Val	V	GUA	GUC	GUG	GUU		
Tryptophan	Trp	W	UGG					
Tyrosine	Tyr	Y	UAC	UAU				

[0160] For instance, inspection of the codon table (Table 1) shows that codons AGA, AGG, CGA, CGC, CGG, and CGU all encode the amino acid arginine. Thus, at every position in the nucleic acids of the invention where an arginine is specified by a codon, the codon can be altered to any of the corresponding codons described above without altering the encoded polypeptide. It is understood that U in an RNA sequence corresponds to T in a DNA sequence.

[0161] Using, as an example, the nucleic acid sequence corresponding to nucleotides 1-15 of SEQ ID NO:1, ATG GCA CTT GCA CCG (SEQ ID NO: 74), a silent variation of this sequence includes ATG GCC TTA GCG CCA (SEQ ID NO: 75), both sequences which encode the amino acid sequence MALAP (SEQ ID NO: 76), corresponding to amino acids 1-5 of SEQ ID NO:26.

[0162] Such "silent variations" are one species of "conservatively modified variations", discussed below. One of skill will recognize that each codon in a nucleic acid (except AUG and UGC, which are ordinarily the only codons for methionine and tryptophan, respectively) can be modified by standard techniques to encode a functionally identical polypeptide. Accordingly, each silent variation of a nucleic acid that encodes a polypeptide is implicit in any described sequence. The invention provides each and every possible variation of nucleic acid sequence encoding a polypeptide of the invention that could be made by selecting combinations based on possible codon choices. These combinations are made in accordance with the standard triplet genetic code (*e.g.*, as set forth in Table 1) as applied to the nucleic acid sequence encoding an FD/FDD homologue polypeptide of the invention or fragments thereof. All such variations of every nucleic acid herein are specifically provided and described by consideration of the sequence in combination with the genetic code. Any variant can be produced as noted herein and one of skill is fully able to generate any silent substitution of the sequences listed herein.

Conservative Variations

[0163] "Conservatively modified variations" or, simply, "conservative variations" of a particular nucleic acid sequence refers to those nucleic acids which encode identical or essentially identical amino acid sequences, or, where the nucleic acid does not encode an amino acid sequence, to essentially identical sequences. One of skill will recognize that individual substitutions, deletions or additions which alter, add or delete a single amino acid or a small percentage of amino acids (typically less than 5%, more typically less than 4%, 3%, 2% or 1% or less) in an encoded sequence are "conservatively modified variations" where the alterations result in the deletion of an amino acid, addition of an amino acid, or substitution of an amino acid with a chemically similar amino acid.

[0164] FD/FDD homologue polypeptides of the present invention include conservatively modified variations of the sequences disclosed herein as SEQ ID NOs:26-50, SEQ ID NOs:70-72, and SEQ ID NOs:85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107, 109, 111, 113, 115, 117, 119, 121, 123, 125, and 127 and fragments thereof. Such conservatively modified variations comprise substitutions, additions or deletions which alter, add or delete a single amino acid or a small percentage of amino acids (typically less than about 5%, more typically less than about 4%, 3%, 2%, or 1%) in any of SEQ ID NOs:26-50, SEQ ID NOs:70-72, and SEQ ID NOs:85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107, 109, 111, 113, 115, 117, 119, 121, 123, 125, and 127.

[0165] Conservative substitution tables providing functionally similar amino acids are well known in the art. Table 2 sets forth six groups which contain amino acids that are "conservative substitutions" for one another.

Table 2

Conservative Substitution Groups

1	Alanine (A)	Serine (S)	Threonine (T)	
2	Aspartic acid (D)	Glutamic acid (E)		
3	Asparagine (N)	Glutamine (Q)		
4	Arginine (R)	Lysine (K)		
5	Isoleucine (I)	Leucine (L)	Methionine (M)	Valine (V)
6	Phenylalanine (F)	Tyrosine (Y)	Tryptophan (W)	

[0166] Thus, "conservatively substituted variations" of a listed polypeptide sequence of the present invention include substitutions of a small percentage, typically less than 5%, more typically less than 4%, 3%, 2% and often less than 1%, of the amino acids of the polypeptide sequence, with a conservatively selected amino acid of the same conservative substitution group.

[0167] For example, a conservatively modified variation (*e.g.*, deletion) of the 600 amino acid polypeptide identified herein as SEQ ID NO:26 will have a length of at least 570 amino acids, preferably at least 576 amino acids, more preferably at least 588 amino acids, and still more preferably at least 594 amino acids, corresponding to a deletion of less than about 5%, 4%, 2% or 1% of the polypeptide sequence. This conservatively modified variation will contain

[0168] Another example of a conservatively modified variation (*e.g.*, a "conservatively substituted variation") of the polypeptide identified herein as SEQ ID NO:26 will contain "conservative substitutions", according to the six substitution groups set forth in Table 2, *supra*, in up to about 30 residues (i.e., less than about 5%) of the 600 amino acid polypeptide.

[0169] In a further example, if four conservative substitutions were localized in the region corresponding to amino acids 1-26 of SEQ ID NO:26, examples of conservatively substituted variations of this region,

MALAP SYINP PNVAS PAGYS HVGVGP (SEQ ID NO: 77) would include:

MAVAP SYINP PQVAS PAGYA HLGVGP (SEQ ID NO: 78) and

MSLAP SWINP PNVAA PAGWS HVGVGP (SEQ ID NO: 79) and the like, in accordance with the conservative substitutions listed in Table 2 (in the above example, conservative substitutions are underlined). Listing of a protein sequence herein, in conjunction with the above substitution table, provides an express listing of all conservatively substituted proteins.

[0170] The FD/FDD polypeptide sequence homologues of the invention, including conservatively substituted sequences, can be present as part of larger polypeptide sequences such as occur upon the addition of one or more domains for purification of the protein (*e.g.*, poly his segments, FLAG tag segments, etc.). These additional functional domains either have little or no effect on the activity of the FD/FDD portion of the protein, or the additional domains can be removed by post synthesis processing steps such as by treatment with a protease, inclusion of an intein or the like.

[0171] A feature of the invention is an FD/FDD homologue polypeptide comprising at least 125 contiguous amino acids of any one of SEQ ID NOs:26-50, SEQ ID NOs:70-72, and SEQ ID NOs:85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107, 109, 111, 113, 115, 117, 119, 121, 123, 125, and 127. In various embodiments, the polypeptide comprises at least about 100, at least about 150, at least about 175, at least about 200, at least about 225, at least about 250, at least about 275, or at least about 300 or more contiguous amino acid residues of any one of SEQ ID NOs:26-50, SEQ ID NOs:70-72 and SEQ ID NOs:85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107, 109, 111, 113, 115, 117, 119, 121, 123, 125, and 127. In some optional embodiments, such fragments optionally comprise a polypeptide with FD/FDD activity (optionally, in yet other embodiments an FD/FDD activity at select pH such at 5.5).

[0172] In other embodiments, the polypeptide is at least 585, 590, or 595 amino acids in length amino acids, preferably at least 598 amino acids, more preferably at least 599 amino acids, and still more preferably at least 600 amino acids in length.

[0173] Finally, the addition of sequences which do not alter the encoded activity of a nucleic acid molecule, such as the addition of a non-functional sequence, is a conservative variation of the basic nucleic acid.

[0174] One of skill will appreciate that many conservative variations of the nucleic acid constructs which are disclosed yield a functionally identical construct. For example, as discussed above, owing to the degeneracy of the genetic code, "silent substitutions" (i.e.,

substitutions in a nucleic acid sequence which do not result in an alteration in an encoded polypeptide) are an implied feature of *every* nucleic acid sequence which encodes an amino acid. Similarly, "conservative amino acid substitutions," in which one or a few amino acids in an amino acid sequence are substituted with different amino acids with highly similar properties, are also readily identified as being highly similar to a disclosed construct. Such conservative variations of each disclosed sequence are a feature of the present invention.

Nucleic Acid Hybridization

[0175] Nucleic acids "hybridize" when they associate, typically in solution. Nucleic acids hybridize due to a variety of well-characterized physico-chemical forces, such as hydrogen bonding, solvent exclusion, base stacking and the like. An extensive guide to the hybridization of nucleic acids is found in Tijssen (1993) *Laboratory Techniques in Biochemistry and Molecular Biology--Hybridization with Nucleic Acid Probes*, part I, chapter 2, "Overview of principles of hybridization and the strategy of nucleic acid probe assays," (Elsevier, New York) (hereinafter "Tijssen"), as well as in Ausubel, Hames and Higgins (1995) *Gene Probes 1,* IRL Press at Oxford University Press, Oxford, England (hereinafter "Hames and Higgins 1") and Hames and Higgins (1995) *Gene Probes 2*, IRL Press at Oxford University Press, Oxford, England (hereinafter "Hames and Higgins 2") provide details on the synthesis, labeling, detection and quantification of DNA and RNA, including oligonucleotides.

[0176] "Stringent hybridization wash conditions" in the context of nucleic acid hybridization experiments, such as Southern and northern hybridizations, are sequence dependent, and are different under different environmental parameters. An extensive guide to the hybridization of nucleic acids is found in Tijssen, Hames and Higgins 1 and Hames and Higgins 2.

[0177] For purposes of the present invention, generally, "highly stringent" hybridization and wash conditions are selected to be about 5° C or less lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength and pH (as noted below, highly stringent conditions can also be referred to in comparative terms). The T_m is the temperature (under defined ionic strength and pH) at which 50% of the test sequence hybridizes to a perfectly matched probe. Very stringent conditions are selected to be equal to the T_m for a particular probe.

[0178] The T_m of the nucleic acid duplexes indicates the temperature at which the duplex is 50% denatured under the given conditions and represents a direct measure of the

stability of the nucleic acid hybrid. Thus, the T_m corresponds to the temperature corresponding to the midpoint in transition from helix to random coil; it depends on length, nucleotide composition, and ionic strength for long stretches of nucleotides.

[0179] After hybridization, unhybridized nucleic acid material can be removed by a series of washes, the stringency of which can be adjusted depending upon the desired results. Low stringency washing conditions (*e.g.*, using higher salt and lower temperature) increase sensitivity, but can produce nonspecific hybridization signals and high background signals. Higher stringency conditions (*e.g.*, using lower salt and higher temperature that is closer to the hybridization temperature) lowers the background signal, typically with only the specific signal remaining. *See*, Rapley, R. and Walker, J.M. eds. (1998), *Molecular Biomethods Handbook,* Humana Press, Inc. (hereinafter "Rapley and Walker"), which is incorporated herein by reference in its entirety for all purposes.

[0180] The T_m of a DNA-DNA duplex can be estimated using the following equation:

$$T_m\ (°C) = 81.5°C + 16.6\ (\log_{10}M) + 0.41\ (\%G + C) - 0.72\ (\%f) - 500/n,$$

where M is the molarity of the monovalent cations (usually Na+), (%G + C) is the percentage of guanosine (G) and cytosine (C) nucleotides, (%f) is the percentage of formamide and n is the number of nucleotide bases (i.e., length) of the hybrid. *See*, Rapley and Walker.

The T_m of an RNA-DNA duplex can be estimated as follows:

$T_m\ (°C) = 79.8°C + 18.5\ (\log_{10}M) + 0.58\ (\%G + C) - 11.8(\%G + C)^2 - 0.56$ (%f) – 820/n, where M is the molarity of the monovalent cations (usually Na+), (%G + C)is the percentage of guanosine (G) and cytosine (C) nucleotides, (%f) is the percentage of formamide and n is the number of nucleotide bases (i.e., length) of the hybrid. *Id.*

[0181] Equations 1 and 2 are typically accurate only for hybrid duplexes longer than about 100-200 nucleotides. *Id.*

[0182] The T_m of nucleic acid sequences shorter than 50 nucleotides can be calculated as follows:

$T_m\ (°C) = 4(G + C) + 2(A + T)$, where A (adenine), C, T (thymine), and G are the numbers of the corresponding nucleotides.

[0183] An example of stringent hybridization conditions for hybridization of complementary nucleic acids which have more than 100 complementary residues on a filter in a Southern or northern blot is 50% formalin with 1 mg of heparin at 42°C, with the hybridization being carried out overnight. An example of stringent wash conditions is a 0.2x SSC wash at 65°C for 15 minutes (*see*, Sambrook for a description of SSC buffer). Often the high stringency wash is preceded by a low stringency wash to remove background probe signal. An example low stringency wash is 2x SSC at 40°C for 15 minutes.

[0184] In general, a signal to noise ratio of 2.5x-5x (or higher) than that observed for an unrelated probe in the particular hybridization assay indicates detection of a specific hybridization. Detection of at least stringent hybridization between two sequences in the context of the present invention indicates relatively strong structural similarity or homology to, *e.g.*, the nucleic acids of the present invention provided in the sequence listings herein.

[0185] As noted, "highly stringent" conditions are selected to be about 5° C or less lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength and pH. Target sequences that are closely related or identical to the nucleotide sequence of interest (*e.g.*, "probe") can be identified under high stringency conditions. Lower stringency conditions are appropriate for sequences that are less complementary. *See*, *e.g.*, Rapley and Walker, *supra*.

[0186] Comparative hybridization can be used to identify nucleic acids of the invention, and this comparative hybridization method is a preferred method of distinguishing nucleic acids of the invention. Detection of highly stringent hybridization between two nucleotide sequences in the context of the present invention indicates relatively strong structural similarity/homology to, *e.g.*, the FD/FDD nucleic acids provided in the sequence listing herein. Highly stringent hybridization between two nucleotide sequences demonstrates a degree of similarity or homology of structure, nucleotide base composition, arrangement or order that is greater than that detected by stringent hybridization conditions. In particular, detection of highly stringent hybridization in the context of the present invention indicates strong structural similarity or structural homology (*e.g.*, nucleotide structure, base composition, arrangement or order) to, *e.g.*, the nucleic acids provided in the sequence listings herein. For example, it is desirable to identify test nucleic acids which hybridize to the exemplar nucleic acids herein under stringent conditions.

[0187] Thus, one measure of stringent hybridization is the ability to hybridize to one of the listed nucleic acids (*e.g.*, nucleic acid sequences SEQ ID NOs:1-25, SEQ ID NOs:67-

69, and SEQ ID NOs:84, 86, 86, 99, 92, 94, 96, 98, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, 120, 122, 124, and 126, and complementary polynucleotide sequences thereof) under highly stringent conditions (or very stringent conditions, or ultra-high stringency hybridization conditions, or ultra-ultra high stringency hybridization conditions). Stringent hybridization (including, *e.g.*, highly stringent, ultra-high stringency, or ultra-ultra high stringency hybridization conditions) and wash conditions can easily be determined empirically for any test nucleic acid.

[0188] For example, in determining highly stringent hybridization and wash conditions, the hybridization and wash conditions are gradually increased (*e.g.*, by increasing temperature, decreasing salt concentration, increasing detergent concentration and/or increasing the concentration of organic solvents, such as formalin, in the hybridization or wash), until a selected set of criteria are met. For example, the hybridization and wash conditions are gradually increased until a probe comprising one or more nucleic acid sequences selected from SEQ ID NOs:1-25, SEQ ID NOs:67-69, and SEQ ID NOs:84, 86, 86, 99, 92, 94, 96, 98, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, 120, 122, 124, and 126, and complementary polynucleotide sequences thereof, binds to a perfectly matched complementary target (again, a nucleic acid comprising one or more nucleic acid sequences selected from SEQ ID NOs:1-25, SEQ ID NOs:67-69, and SEQ ID NOs:84, 86, 86, 99, 92, 94, 96, 98, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, 120, 122, 124, and 126, and complementary polynucleotide sequences thereof), with a signal to noise ratio that is at least 2.5x, and optionally 5x or more as high as that observed for hybridization of the probe to an unmatched target. In this case, the unmatched target is a nucleic acid corresponding to, *e.g.*, a known FD/FDD homologue, such as an FD/FDD homologue nucleic acid that is present in a public database such as GenBank™ at the time of filing of the subject application. Examples of such unmatched target nucleic acids include, *e.g.*, nucleic acids encoding ESP002C2, ESP002C3, ESP003C12, RAT011C1, RAT011C2, RAT011C4 (*see*, SEQ ID Nos: 51, 53, 55, 57, 59, 61 and 63) where the clone identification numbers correspond to those in PCT publications WO 00/04159 and WO 00/04160, as well as the nucleic acid sequence encoding wild type APAO (SEQ ID NO:52). Additional such sequences can be identified in, *e.g.*, GenBank by one of ordinary skill in the art.

[0189] A test nucleic acid is said to specifically hybridize to a probe nucleic acid when it hybridizes at least one-half as well to the probe as to the perfectly matched complementary target, i.e., with a signal to noise ratio at least one-half as high as hybridization of the probe to the target under conditions in which the perfectly matched probe binds to the perfectly matched complementary target with a signal to noise ratio that is at least

about 2.5x-10x, typically 5x-10x as high as that observed for hybridization to any of the unmatched target nucleic acids such as, nucleic acids encoding ESP002C2, ESP002C3, ESP003C12, RAT011C1, RAT011C2, RAT011C4, where the clone identification numbers correspond to those in PCT publications WO 00/04159 and WO 00/04160, or encoding wild type APAO (SEQ ID NO:51) or, *e.g.*, other similar FD/FDD sequences presented in, *e.g.*, GenBank.

[0190] Ultra high-stringency hybridization and wash conditions are those in which the stringency of hybridization and wash conditions are increased until the signal to noise ratio for binding of the probe to the perfectly matched complementary target nucleic acid is at least 10x as high as that observed for hybridization to any of the unmatched target nucleic acids, such as, nucleic acids encoding ESP002C2, ESP002C3, ESP003C12, RAT011C1, RAT011C2, RAT011C4 (where the numbers correspond to clone numbers in PCT publications WO 00/04159 and WO 00/04160), or the nucleic acid encoding wild type APAO (SEQ ID NO:51) or, *e.g.*, to other similar FD/FDD molecule sequences presented in, *e.g.*, GenBank. A target nucleic acid which hybridizes to a probe under such conditions, with a signal to noise ratio of at least one-half that of the perfectly matched complementary target nucleic acid is said to bind to the probe under ultra-high stringency conditions.

[0191] Similarly, even higher levels of stringency can be determined by gradually increasing the hybridization and/or wash conditions of the relevant hybridization assay. For example, those in which the stringency of hybridization and wash conditions are increased until the signal to noise ratio for binding of the probe to the perfectly matched complementary target nucleic acid is at least 10x, 20X, 30X, 40X, 50X, 75X, 100X, 200X, 300X, 400X, or 500X or more as high as that observed for hybridization to any of the unmatched target nucleic acids, such as those represented by: ESP002C2, ESP002C3, ESP003C12, RAT011C1, RAT011C2, RAT011C4 (where the numbers correspond to clone numbers in patents W/O 00/04159 and W/O 00/04160), or wild-type APAO or, *e.g.*, other similar FD/FDD sequences presented in, *e.g.*, GenBank can be identified. A target nucleic acid which hybridizes to a probe under such conditions, with a signal to noise ratio of at least one-half that of the perfectly matched complementary target nucleic acid is said to bind to the probe under ultra-ultra-high stringency conditions.

[0192] Target nucleic acids which hybridize to the nucleic acids represented by SEQ ID NOs:1-25, SEQ ID NOs:67-69, and SEQ ID NOs:84, 86, 86, 99, 92, 94, 96, 98, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, 120, 122, 124, and 126 under high, ultra-high and ultra-ultra high stringency conditions are a feature of the invention. Examples of such nucleic

acids include those with one or a few silent or conservative nucleic acid substitutions as compared to a given nucleic acid sequence.

[0193] Nucleic acids which do not hybridize to each other under stringent conditions are still substantially identical if the polypeptides which they encode are substantially identical. This occurs, *e.g.*, when a copy of a nucleic acid is created using the maximum codon degeneracy permitted by the genetic code, or when antisera generated against one or more of SEQ ID NOs:26-50, SEQ ID NOs:70-72, and SEQ ID NOs:85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107, 109, 111, 113, 115, 117, 119, 121, 123, 125, and 127 which has been subtracted using the polypeptides encoded by known or existing FD/FDD sequences, including, *e.g.*, those encoded by the following: ESP002C2, ESP002C3, ESP003C12, RAT011C1, RAT011C2, RAT011C4 (where the numbers correspond to clone numbers in PCT publications WO 00/04159 and WO 00/04160), or wild type APAO (SEQ ID NO:52) or, *e.g.*, other similar FD/FDD sequences presented in, *e.g.*, GenBank. Further details on immunological identification of polypeptides of the invention are found below. Additionally, for distinguishing between duplexes with sequences of less than about 100 nucleotides, a TMAC1 hybridization procedure known to those of ordinary skill in the art can be used. *See, e.g.*, Sorg, U. *et al. Nucleic Acids Res.* (1991) 19:4782, incorporated herein by reference in its entirety for all purposes.

[0194] In one aspect, the invention provides a nucleic acid which comprises a unique subsequence in a nucleic acid selected from SEQ ID NOs:1-25, SEQ ID NOs:67-69, and SEQ ID NOs:84, 86, 86, 99, 92, 94, 96, 98, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, 120, 122, 124, and 126. The unique subsequence is unique as compared to a nucleic acid corresponding to any of, *e.g.*, ESP002C2, ESP002C3, ESP003C12, RAT011C1, RAT011C2, RAT011C4 (where the numbers correspond to clone numbers in PCT publications WO 00/04159 and WO 00/04160), or wild type APAO (SEQ ID NO:51) or, *e.g.*, other similar FD/FDD sequences presented in, *e.g.*, GenBank. Such unique subsequences can be determined by aligning any of SEQ ID NOs:1-25, SEQ ID NOs:67-69, and SEQ ID NOs:84, 86, 86, 99, 92, 94, 96, 98, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, 120, 122, 124, and 126 against the complete set of nucleic acids, *e.g.*, those corresponding to, *e.g.*, nucleic acids encoding ESP002C2, ESP002C3, ESP003C12, RAT011C1, RAT011C2, RAT011C4, or the nucleic acid encoding wild type APAO (SEQ ID NO:51) or other sequences available, *e.g.*, in a public database, at the filing date of the subject application. Alignment can be performed using the BLAST algorithm set to default parameters. Any unique subsequence is useful, *e.g.*, as a probe to identify the nucleic acids of the invention.

[0195] Similarly, the invention includes a polypeptide which comprises a unique amino acid subsequence of a polypeptide selected from SEQ ID NOs:26-50, SEQ ID NOs:70-72, and SEQ ID NOs:85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107, 109, 111, 113, 115, 117, 119, 121, 123, 125, and 127. Here, the unique subsequence is unique as compared to a polypeptide or amino acid sequence corresponding to, *e.g.*, any of ESP002C2, ESP002C3, ESP003C12, RAT011C1, RAT011C2, RAT011C4 (where the numbers correspond to clone numbers in PCT publications WO 00/04159 and WO 00/04160) or the wild type APAO (SEQ ID NO:52). Here again, the polypeptide is aligned against the existing polypeptides (the control polypeptides). Note that where the sequence corresponds to a non-translated sequence such as a pseudo-gene, the corresponding polypeptide is generated simply by in silico translation of the nucleic acid sequence into an amino acid sequence, where the reading frame is selected to correspond to the reading frame of homologous FD/FDD nucleic acids. Such polypeptides are optionally made by synthetic or recombinant approaches, or can even be ordered from companies specializing in polypeptide production.

[0196] In addition, the present invention provides a target nucleic acid which hybridizes under at least stringent or highly stringent conditions (or conditions of greater stringency) to a unique coding oligonucleotide which encodes a unique subsequence in a polypeptide selected from: SEQ ID NOs:26-50, SEQ ID NOs:70-72, and SEQ ID NOs:85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107, 109, 111, 113, 115, 117, 119, 121, 123, 125, and 127, wherein the unique subsequence is unique as compared to an amino acid subsequence of a known FD/FDD polypeptide sequence shown in, *e.g.*, GenBank or to a polypeptide corresponding to any of the control polypeptides (*see*, above). Unique sequences are determined as noted above.

[0197] In one example, the stringent conditions are selected such that a perfectly complementary oligonucleotide to the coding oligonucleotide hybridizes to the coding oligonucleotide with at least about a 5-10x higher signal to noise ratio than for hybridization of the perfectly complementary oligonucleotide to a control nucleic acid corresponding to any of the control polypeptides. Conditions can be selected such that higher ratios of signal to noise are observed in the particular assay which is used, *e.g.*, about 15x, 20x, 30x, 50x or more. In this example, the target nucleic acid hybridizes to the unique coding oligonucleotide with at least a 2x higher signal to noise ratio as compared to hybridization of the control nucleic acid to the coding oligonucleotide. Again, higher signal to noise ratios can be selected, *e.g.*, about 2.5x, about 5x, about 10x, about 20x, about 30x, about 50x or more. The particular signal will depend on the label used in the relevant assay, *e.g.*, a fluorescent label, a colorimetric label, a radioactive label, or the like.

[0198] In another aspect, the invention provides a polypeptide that comprises a unique subsequence in a polypeptide selected from SEQ ID NOs:26-50, SEQ ID NOs:70-72, and SEQ ID NOs:85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107, 109, 111, 113, 115, 117, 119, 121, 123, 125, and 127, wherein the unique subsequence is unique as compared to a polypeptide sequence corresponding to a known FD/FDD polypeptide, such as, *e.g.*, an FD/FDD polypeptide sequence present in GenBank.

Percent Sequence Identity – Sequence Similarity

[0199] As noted above, the peptides employed in the subject invention need not be identical, but can be substantially identical, to the corresponding sequence of an FD/FDD molecule or related molecule. The peptides can be subject to various changes, such as insertions, deletions, and substitutions, either conservative or non-conservative, where such changes might provide for certain advantages in their use. The polypeptides of the invention can be modified in a number of ways so long as they comprise a sequence substantially identical (as defined below) to a sequence in an FD/FDD molecule.

[0200] Alignment and comparison of relatively short amino acid sequences (less than about 30 residues) is typically straightforward. Comparison of longer sequences can require more sophisticated methods to achieve optimal alignment of two sequences. Optimal alignment of sequences for aligning a comparison window can be conducted by the local homology algorithm of Smith and Waterman (1981) *Adv Appl Math* 2:482, by the homology alignment algorithm of Needleman and Wunsch (1970) *J Mol Biol* 48:443, by the search for similarity method of Pearson and Lipman (1988) *PNAS USA* 85:2444, by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package Release 7.0, Genetics Computer Group, 575 Science Dr., Madison, WI), or by inspection, with the best alignment (i.e., resulting in the highest percentage of sequence similarity over the comparison window) generated by the various methods being selected.

[0201] The term sequence identity means that two polynucleotide sequences are identical (i.e., on a nucleotide-by-nucleotide basis) over a window of comparison. The term "percentage of sequence identity" or "percent sequence identity" is calculated by comparing two optimally aligned sequences over the window of comparison, determining the number of positions at which the identical residues occur in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison (i.e., the window size), and multiplying the result by 100 to yield the percentage of sequence identity. In one aspect, the present invention provides

FD/FDD homologue nucleic acids having at least about 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%. 99.5% or more sequence identity with the nucleic acids of any of SEQ ID NOs:1-25, SEQ ID NOs:67-69, and SEQ ID NOs:84, 86, 86, 99, 92, 94, 96, 98, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, 120, 122, 124, and 126 or fragments thereof. In other embodiments the invention provides FD/FDD homologue polypeptides with an ability to detoxify fumonisins or fumonisin-derivatives wherein the polypeptide has at least a 70%, at least a 75%, at least an 80%, at least an 85%, at least 90%, at least a 91%, at least a 92%, at least a 93%, at least a 94%, at least a 95%, at least a 96%, at least a 97%, at least a 98%, at least a 99%, or at least 99.5% or more identity to at least one of SEQ ID NOs:26-50, SEQ ID NOs:70-72, and SEQ ID NOs:85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107, 109, 111, 113, 115, 117, 119, 121, 123, 125, and 127 over a comparison window of at least 100, at least 110, at least 115, at least 120, at least 125, at least 130, at least 135, at least 140, at least 150, at least 175, at least 200, at least 225, at least 250, or at least 275 or more contiguous amino acids. In some optional embodiments, the above percent identities over the listed contiguous amino acid lengths apply to FD/FDD homologues of the invention which possess at least partial fumonisin detoxification ability and/or which possess a pH optimum in a pH range of from about 5.0 to about 7.4.

[0202] As applied to polypeptides, the term substantial identity means that two peptide sequences, when optimally aligned, such as by the programs GAP or BESTFIT using default gap weights (described in detail below), share at least about 80 percent sequence identity, preferably at least about 90 percent sequence identity, more preferably at least about 95 percent sequence identity or more (*e.g.*, 96, 97, 98, 99, or 99.5 or more percent sequence identity). Alternatively, parameters are set such that one or more sequences of the invention, *e.g.*, SEQ ID NOs:26-50, SEQ ID NOs:70-72, and SEQ ID NOs:85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107, 109, 111, 113, 115, 117, 119, 121, 123, 125, and 127 are identified by alignment to a query sequence selected from among SEQ ID NOs:26-50, SEQ ID NOs:70-72, and SEQ ID NOs:85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107, 109, 111, 113, 115, 117, 119, 121, 123, 125, and 127, while sequences corresponding to unrelated polypeptides, *e.g.*, those corresponding to clone numbers ESP002C2, ESP002C3, ESP003C12, RAT011C1, RAT011C2, RAT011C4 from PCT publications WO 00/04159 and WO00/04160 or wild-type APAO (SEQ ID NO:52) or other similar fumonisin detoxification molecules found in, *e.g.*, GenBank, are not identified.

[0203] Preferably, residue positions which are not identical differ by conservative amino acid substitutions. Conservative amino acid substitution refers to the interchangeability of residues having similar side chains. For example, a group of amino

acids having aliphatic side chains is alanine, valine, leucine, and isoleucine, and also includes glycine; a group of amino acids having aliphatic-hydroxyl side chains is serine and threonine; a group of amino acids having amide-containing side chains is asparagine and glutamine; a group of amino acids having aromatic side chains is phenylalanine, tyrosine, and tryptophan; a group of amino acids having basic side chains is lysine, arginine, and histidine; and a group of amino acids having sulfur-containing side chains is cysteine and methionine. Preferred conservative amino acids substitution groups are: valine-leucine-isoleucine, phenylalanine-tyrosine, lysine-arginine, alanine-valine, and asparagine-glutamine. In one aspect, the present invention provides FD/FDD homologue polypeptides having at least about 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, 99.5%, or more percent sequence identity with the polypeptides of any of SEQ ID NOs:26-50, SEQ ID NOs:70-72, and SEQ ID NOs:85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107, 109, 111, 113, 115, 117, 119, 121, 123, 125, and 127.

[0204] A preferred example of an algorithm that is suitable for determining percent sequence identity and sequence similarity is the FASTA algorithm, which is described in Pearson, W.R. & Lipman, D. J., (1988) *PNAS USA* 85:2444. *See* also, W. R. Pearson, (1996) *Methods Enzymol* 266:227-258. Preferred parameters used in a FASTA alignment of DNA sequences to calculate percent identity are optimized, BL50 Matrix 15: -5, k-tuple = 2; joining penalty = 40, optimization = 28; gap penalty -12, gap length penalty =-2; and width = 16.

[0205] Other preferred examples of algorithm that are suitable for determining percent sequence identity and sequence similarity are the BLAST and BLAST 2.0 algorithms, which are described in Altschul *et al.*, (1977) *Nuc Acids Res* 25:3389-3402 and Altschul *et al.*, (1990) *J Mol Biol* 215:403-410, respectively. BLAST and BLAST 2.0 are used, with the parameters described herein, to determine percent sequence identity for the nucleic acids and proteins of the invention. Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/). This algorithm involves first identifying high scoring sequence pairs (HSPs) by identifying short words of length 'W' in the query sequence, which either match or satisfy some positive-valued threshold score 'T' when aligned with a word of the same length in a database sequence. T is referred to as the neighborhood word score threshold (Altschul). These initial neighborhood word hits act as seeds for initiating searches to find longer HSPs containing them. The word hits are extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Cumulative scores are calculated using, for nucleotide sequences, the parameters 'M' (reward score for a pair of matching residues; always > 0) and 'N' (penalty score for mismatching residues; always < 0). For amino acid sequences, a scoring matrix is used to calculate the cumulative score. Extension of the word

hits in each direction are halted when: the cumulative alignment score falls off by the quantity 'X' from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters W, T, and X determine the sensitivity and speed of the alignment. The BLASTN program (for nucleotide sequences) uses as defaults a wordlength (W) of 11, an expectation (E) of 10, M=5, N=-4 and a comparison of both strands. For amino acid sequences, the BLASTP program uses as defaults a wordlength of 3, and expectation (E) of 10, and the BLOSUM62 scoring matrix (*see*, Henikoff & Henikoff, (1989) *PNAS USA* 89:10915) uses alignments (B) of 50, expectation (E) of 10, M=5, N=-4, and a comparison of both strands. Again, as with other suitable algorithms, the stringency of comparison can be increased until the program identifies only sequences that are more closely related to those in the sequence listings herein (i.e., SEQ ID NOs:1-25, SEQ ID NOs:67-69, and SEQ ID NOs:84, 86, 86, 99, 92, 94, 96, 98, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, 120, 122, 124, and 126 or, alternatively, SEQ ID NOs:26-50, SEQ ID NOs:70-72, and SEQ ID NOs:85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107, 109, 111, 113, 115, 117, 119, 121, 123, 125, and 127), than to sequences that are more closely related to other sequences such as, *e.g.*, clone numbers ESP002C2, ESP002C3, ESP003C12, RAT011C1, RAT011C2, RAT011C4 from PCT publications WO 00/04159 and WO00/04160 or wild-type APAO (SEQ ID NOs:51-52) or similar molecules found in, *e.g.*, GenBank . In other words, the stringency of comparison of the algorithms can be increased so that all known prior art (*e.g.*, clone numbers ESP002C2, ESP002C3, ESP003C12, RAT011C1, RAT011C2, RAT011C4 from PCT publications WO 00/04159 and WO 00/04160) or wild-type APAO (SEQ ID NOs:51 and 52) or other similar molecules found in, *e.g.*, GenBank) is excluded.

[0206] The BLAST algorithm also performs a statistical analysis of the similarity between two sequences (*see*, *e.g.*, Karlin & Altschul, (1993) *PNAS USA* 90:5873-5787). One measure of similarity provided by the BLAST algorithm is the smallest sum probability (P(N)), which provides an indication of the probability by which a match between two nucleotide or amino acid sequences would occur by chance. For example, a nucleic acid is considered similar to a reference sequence if the smallest sum probability in a comparison of the test nucleic acid to the reference nucleic acid is less than about 0.2, more preferably less than about 0.01, and most preferably less than about 0.001.

[0207] Another example of a useful algorithm is PILEUP. PILEUP creates a multiple sequence alignment from a group of related sequences using progressive, pairwise alignments to show relationship and percent sequence identity. It also plots a tree or

dendrogram showing the clustering relationships used to create the alignment. PILEUP uses a simplification of the progressive alignment method of Feng & Doolittle, (1987) *J Mol Evol* 35:351-360. The method used is similar to the method described by Higgins & Sharp, (1989) *CABIOS* 5:151-153. The program can align up to 300 sequences, each of a maximum length of 5,000 nucleotides or amino acids. The multiple alignment procedure begins with the pairwise alignment of the two most similar sequences, producing a cluster of two aligned sequences. This cluster is then aligned to the next most related sequence or cluster of aligned sequences. Two clusters of sequences are aligned by a simple extension of the pairwise alignment of two individual sequences. The final alignment is achieved by a series of progressive, pairwise alignments. The program is run by designating specific sequences and their amino acid or nucleotide coordinates for regions of sequence comparison and by designating the program parameters. Using PILEUP, a reference sequence is compared to other test sequences to determine the percent sequence identity relationship using the following parameters: default gap weight (3.00), default gap length weight (0.10), and weighted end gaps. PILEUP can be obtained from the GCG sequence analysis software package, *e.g.*, version 7.0 (Devereaux *et al.*, (1984) *Nuc Acids Res* 12:387-395).

[0208] Another preferred example of an algorithm that is suitable for multiple DNA and amino acid sequence alignments is the CLUSTALW program (Thompson, J. D. *et al.*, (1994) *Nuc Acids Res* 22:4673-4680). CLUSTALW performs multiple pairwise comparisons between groups of sequences and assembles them into a multiple alignment based on homology. Gap open and Gap extension penalties were 10 and 0.05 respectively. For amino acid alignments, the BLOSUM algorithm can be used as a protein weight matrix (Henikoff and Henikoff, (1992) *PNAS USA* 89:10915-10919).

[0209] It will be understood by one of ordinary skill in the art, that the above discussion of search and alignment algorithms also applies to identification and evaluation of polynucleotide sequences, with the substitution of query sequences comprising nucleotide sequences, and where appropriate, selection of nucleic acid databases.

SUBSTRATES AND FORMATS FOR SEQUENCE RECOMBINATION

[0210] The polynucleotides of the invention and fragments thereof are optionally used as substrates for a variety of recombination and recursive recombination reactions, in addition to standard cloning methods as set forth in, *e.g.*, Ausubel, Berger and Sambrook, i.e., to produce additional FD/FDD homologues and fragments thereof with desired properties. A variety of such reactions are known, including those developed by the inventors and their co-workers. Methods for producing a variant of any nucleic acid of the invention listed herein

comprising recursively recombining such polynucleotide with a second (or more) polynucleotide, thus forming a library of variant polynucleotides are also features of the invention, as are the libraries produced, the cells comprising the libraries, and any recombinant polynucleotide produces by such methods. Additionally, such methods optionally comprise selecting a variant polynucleotide from such libraries based on FD/FDD activity, as is wherein such recursive recombination is done in vitro or *in vivo*.

[0211] A variety of diversity generating protocols, including nucleic acid recursive recombination protocols, is available and fully described in the art. The procedures can be used separately, and/or in combination to produce one or more variants of a nucleic acid or set of nucleic acids, as well variants of encoded proteins. Individually and collectively, these procedures provide robust, widely applicable ways of generating diversified nucleic acids and sets of nucleic acids (including, *e.g.*, nucleic acid libraries) useful, *e.g.*, for the engineering or rapid evolution of nucleic acids, proteins, pathways, cells and/or organisms with new and/or improved characteristics.

[0212] While distinctions and classifications are made in the course of the ensuing discussion for clarity, it will be appreciated that the techniques are often not mutually exclusive. Indeed, the various methods can be used singly or in combination, in parallel or in series, to access diverse sequence variants.

[0213] The result of any of the diversity generating procedures described herein can be the generation of one or more nucleic acids, which can be selected or screened for nucleic acids with or which confer desirable properties, or that encode proteins with or which confer desirable properties. Following diversification by one or more of the methods herein, or otherwise available to one of skill, any nucleic acids that are produced can be selected for a desired activity or property, *e.g.* FD/FDD activity, or, such activity at a desired pH, etc. This can include identifying any activity that can be detected, for example, in an automated or automatable format, by any of the assays in the art, *see*, *e.g.*, discussion of screening of FD/FDD activity, *infra*. A variety of related (or even unrelated) properties can be evaluated, in serial or in parallel, at the discretion of the practitioner.

[0214] Descriptions of a variety of diversity generating procedures for generating modified nucleic acid sequences, *e.g.*, those coding for polypeptides having FD/FDD activity, or fragments thereof, are found in the following publications and the references cited therein: Soong, N. *et al.* (2000) *Nat Genet* 25(4):436-439; Stemmer, *et al.* (1999) *Tumor Targeting* 4:1-4; Ness *et al.* (1999) *Nat Biotechnol* 17:893-896; Chang *et al.* (1999) *Nat Biotechnol* 17:793-797; Minshull and Stemmer (1999) *Curr Opin Chem Biol* 3:284-290; Christians *et al.*

(1999) *Nat Biotechnol* 17:259-264; Crameri *et al.* (1998) *Nature* 391:288-291; Crameri *et al.* (1997) *Nat Biotechnol* 15:436-438; Zhang *et al.* (1997) *PNAS USA* 94:4504-4509; Patten *et al.* (1997) *Curr Opin Biotechnol* 8:724-733; Crameri *et al.* (1996) *Nat Med* 2:100-103; Crameri *et al.* (1996) *Nat Biotechnol* 14:315-319; Gates *et al.* (1996) *J Mol Biol* 255:373-386; Stemmer (1996) "Sexual PCR and Assembly PCR" In: *The Encyclopedia of Molecular Biology.* VCH Publishers, New York. pp.447-457; Crameri and Stemmer (1995) *BioTechniques* 18:194-195; Stemmer *et al.*, (1995) *Gene*, 164:49-53; Stemmer (1995) *Science* 270: 1510; Stemmer (1995) *Bio/Technology* 13:549-553; Stemmer (1994) *Nature* 370:389-391; and Stemmer (1994) *PNAS USA* 91:10747-10751.

[0215] Mutational methods of generating diversity include, for example, site-directed mutagenesis (Ling *et al.* (1997) *Anal Biochem* 254(2): 157-178; Dale *et al.* (1996) *Methods Mol Biol* 57:369-374; Smith (1985) *Ann Rev Genet* 19:423-462; Botstein & Shortle (1985) *Science* 229:1193-1201; Carter (1986) *Biochem J* 237:1-7; and Kunkel (1987) "The efficiency of oligonucleotide directed mutagenesis" in *Nucleic Acids & Molecular Biology* (Eckstein, F. and Lilley, D.M.J. eds., Springer Verlag, Berlin)); mutagenesis using uracil containing templates (Kunkel (1985) *PNAS USA* 82:488-492; Kunkel *et al.* (1987) *Methods Enzymol* 154, 367-382; and Bass *et al.* (1988) *Science* 242:240-245); oligonucleotide-directed mutagenesis (Zoller & Smith (1983) *Methods Enzymol* 100: 468-500; Zoller & Smith (1987) *Methods Enzymol* 154: 329-350 (1987); Zoller & Smith (1982) *Nucleic Acids Res* 10:6487-6500), phosphorothioate-modified DNA mutagenesis (Taylor *et al.* (1985) *Nucl Acids Res* 13: 8749-8764; Taylor *et al.* (1985) *Nucl Acids Res* 13: 8765-8787 (1985); Nakamaye & Eckstein (1986) *Nucl Acids Res* 14: 9679-9698; Sayers *et al.* (1988) *Nucl Acids Res* 16:791-802; and Sayers *et al.* (1988) *Nucl Acids Res* 16: 803-814); mutagenesis using gapped duplex DNA (Kramer *et al.* (1984) *Nucl Acids Res* 12: 9441-9456; Kramer & Fritz (1987) *Methods Enzymol* 154:350-367; Kramer *et al.* (1988) *Nucl Acids Res* 16: 7207; and Fritz *et al.* (1988) *Nucl Acids Res* 16: 6987-6999).

[0216] Additional suitable methods include point mismatch repair (Kramer *et al.* (1984) *Cell* 38:879-887), mutagenesis using repair-deficient host strains (Carter *et al.* (1985) *Nucl Acids Res* 13: 4431-4443; and Carter (1987) *Methods in Enzymol* 154: 382-403), deletion mutagenesis (Eghtedarzadeh & Henikoff (1986) *Nucl Acids Res* 14: 5115), restriction-selection and restriction-purification (Wells *et al.* (1986) *Phil Trans R Soc Lond* A 317: 415-423), mutagenesis by total gene synthesis (Nambiar *et al.* (1984) *Science* 223: 1299-1301; Sakamar and Khorana (1988) *Nucl Acids Res* 14: 6361-6372; Wells *et al.* (1985) *Gene* 34:315-323; and Grundström *et al.* (1985) *Nucl Acids Res* 13: 3305-3316), double-strand break repair (Mandecki (1986) *PNAS USA*, 83:7177-7181; and Arnold (1993) *Curr Opin*

Biotech 4:450-455). Additional details on many of the above methods can be found in *Methods Enzymol* Volume 154, which also describes useful controls for trouble-shooting problems with various mutagenesis methods.

[0217] Additional details regarding various diversity generating methods can be found in the following U.S. patents, PCT publications and applications, and EPO publications: U.S. Pat. No. 5,723,323, U.S. Pat. No. 5,763,192, U.S. Pat. No. 5,814,476, U.S. Pat. No. 5,817,483, U.S. Pat. No. 5,824,514, U.S. Pat. No. 5,976,862, U.S. Pat. No. 5,605,793, U.S. Pat. No. 5,811,238, U.S. Pat. No. 5,830,721, U.S. Pat. No. 5,834,252, U.S. Pat. No. 5,837,458, WO 95/22625, WO 96/33207, WO 97/20078, WO 97/35966, WO 99/41402, WO 99/41383, WO 99/41369, WO 99/41368, EP 752008, EP 0932670, WO 99/23107, WO 99/21979, WO 98/31837, WO 98/27230, WO 98/27230, WO 00/00632, WO 00/09679, WO 98/42832, WO 99/29902, WO 98/41653, WO 98/41622, WO 98/42727, WO 00/18906, WO 00/04190, WO 00/42561, WO 00/42559, WO 00/42560, WO 01/23401, and PCT/US01/06775.

[0218] In brief, several different general classes of sequence modification methods, such as mutation, recombination, etc., are applicable to the present invention (i.e., to generate FD/FDD homologues) and are set forth, *e.g.*, in the references above.

[0219] The following exemplify some of the different types of preferred formats for diversity generation in the context of the present invention, including, *e.g.*, certain recombination based diversity generation formats.

[0220] Nucleic acids can be recombined in vitro by any of a variety of techniques discussed in the references above, including *e.g.*, DNAse digestion of nucleic acids to be recombined followed by ligation and/or PCR reassembly of the nucleic acids. For example, sexual PCR mutagenesis can be used in which random (or pseudo random, or even non-random) fragmentation of the DNA molecule is followed by recombination, based on sequence similarity, between DNA molecules with different but related DNA sequences, in vitro, followed by fixation of the crossover by extension in a polymerase chain reaction. This process and many process variants are described in several of the references above, *e.g.*, in Stemmer (1994) *PNAS USA* 91:10747-10751. Thus FD/FDD homologues of the invention are optionally generated through such methods, or optionally are used in such methods as starting points for generation of further diversity.

[0221] Similarly, nucleic acids can be recursively recombined *in vivo*, *e.g.*, by allowing recombination to occur between nucleic acids in cells. Many such *in vivo*

recombination formats are set forth in the references noted above. Such formats optionally provide direct recombination between nucleic acids of interest, or provide recombination between vectors, viruses, plasmids, etc., comprising the nucleic acids of interest, as well as other formats. Details regarding such procedures are found in the references noted above. Thus, again, the FD/FDD homologues of the invention are optionally generated through such recursive recombination techniques, or optionally are used in such methods as starting points for generation of further diversity (i.e., to generate, *e.g.*, additional FD/FDD homologues).

[0222] Whole genome recombination methods can also be used in which whole genomes of cells or other organisms are recombined, optionally including spiking of the genomic recombination mixtures with desired library components (*e.g.*, genes corresponding to the pathways of the present invention, i.e., fumonisin detoxification, etc.). These methods have many applications, including those in which the identity of a target gene is not known. Details on such methods are found, *e.g.*, in WO 98/31837 and WO 00/04190.

[0223] Synthetic recombination methods can also be used, in which oligonucleotides corresponding to targets of interest are synthesized and reassembled in PCR or ligation reactions which include oligonucleotides which correspond to more than one parental nucleic acid, thereby generating new recombined nucleic acids. Oligonucleotides can be made by standard nucleotide addition methods, or can be made, *e.g.*, by tri-nucleotide synthetic approaches. Details regarding such approaches are found in the references noted above, including, *e.g.*, WO 00/42561, WO 01/23401, WO 00/42560, and WO 00/42559.

[0224] In silico methods of recombination can be effected in which genetic algorithms are used in a computer to recombine sequence strings which correspond to homologous (or even non-homologous) nucleic acids. The resulting recombined sequence strings are optionally converted into nucleic acids by synthesis of nucleic acids which correspond to the recombined sequences, *e.g.*, in concert with oligonucleotide synthesis/gene reassembly techniques. This approach can generate random, partially random or designed variants. Many details regarding in silico recombination, including the use of genetic algorithms, genetic operators and the like in computer systems, combined with generation of corresponding nucleic acids (and/or proteins), as well as combinations of designed nucleic acids and/or proteins (*e.g.*, based on cross-over site selection) as well as designed, pseudo-random or random recombination methods are described in WO 00/42560 and WO 00/42559. Extensive details regarding in silico recombination methods are found in these applications. This methodology is generally applicable to the present invention in providing for

recombination of the FD/FDD homologues in silico and/or the generation of corresponding nucleic acids or proteins.

[0225] Many methods of accessing natural diversity, *e.g.*, by hybridization of diverse nucleic acids or nucleic acid fragments to single-stranded templates, followed by polymerization and/or ligation to regenerate full-length sequences, optionally followed by degradation of the templates and recovery of the resulting modified nucleic acids can be similarly used. In one method employing a single-stranded template, the fragment population derived from the genomic library(ies) is annealed with partial, or, often approximately full length ssDNA or RNA corresponding to the opposite strand. Assembly of complex chimeric genes from this population is then mediated by nuclease-base removal of non-hybridizing fragment ends, polymerization to fill gaps between such fragments and subsequent single stranded ligation. The parental polynucleotide strand can be removed by digestion (*e.g.*, if RNA or uracil-containing), magnetic separation under denaturing conditions (if labeled in a manner conducive to such separation) and other available separation/purification methods. Alternatively, the parental strand is optionally co-purified with the chimeric strands and removed during subsequent screening and processing steps. Additional details regarding this approach are found, *e.g.*, in PCT/US01/06775.

[0226] In another approach, single-stranded molecules are converted to double-stranded DNA (dsDNA) and the dsDNA molecules are bound to a solid support by ligand-mediated binding. After separation of unbound DNA, the selected DNA molecules are released from the support and introduced into a suitable host cell to generate a library of enriched sequences which hybridize to the probe. A library produced in this manner provides a desirable substrate for further diversification using any of the procedures described herein.

[0227] Any of the preceding general recombination formats can be practiced in a reiterative fashion (*e.g.*, one or more cycles of mutation/recombination or other diversity generation methods, optionally followed by one or more selection methods) to generate a more diverse set of recombinant nucleic acids.

[0228] The above references provide these and other basic recombination formats as well as many modifications of these formats. Regardless of the format which is used, the nucleic acids of the invention can be recombined (with each other, or with related (or even unrelated) sequences) to produce a diverse set of recombinant nucleic acids, including, *e.g.*, sets of homologous nucleic acids. In general, the sequence recombination techniques described herein provide particular advantages in that they provide for recombination between the nucleic acids of SEQ ID NOs:1-25, SEQ ID NOs:67-69, and SEQ ID NOs:84, 86, 86, 99,

92, 94, 96, 98, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, 120, 122, 124, and 126, or derivatives thereof, in any available format, thereby providing a very fast way of exploring the manner in which different combinations of sequences can affect a desired result.

[0229] Following recombination, any nucleic acids which are produced can be selected for a desired activity. In the context of the present invention, this can include testing for and identifying any activity that can be detected, *e.g.*, any of the usual FD/FDD activities, by any of the assays in the art, *e.g.*, in an automatable format. A variety of related (or even unrelated) properties can be assayed for, using any available assay.

[0230] A recombinant nucleic acid produced by recursively recombining one or more polynucleotide of the invention with one or more additional nucleic acid also forms a part of the invention. The one or more additional nucleic acid may include another polynucleotide of the invention; optionally, alternatively, or in addition, the one or more additional nucleic acid can include, *e.g.*, a nucleic acid encoding a naturally-occurring FD/FDD homologue or a subsequence thereof, or any homologous FD/FDD sequence or subsequence thereof (*e.g.*, as found in GenBank or other available literature), or, *e.g.*, any other homologous or non-homologous nucleic acid (certain recombination formats noted above, notably those performed synthetically or in silico, do not require homology for recombination).

[0231] The recombining steps may be performed *in vivo*, in vitro, in planta, or in silico as described in more detail in the references above. Also included in the invention is a cell containing any resulting recombinant nucleic acid, nucleic acid libraries produced by recursive recombination of the nucleic acids set forth herein, and populations of cells, vectors, viruses, plasmids or the like comprising the library or comprising any recombinant nucleic acid resulting from recombination (or recursive recombination) of a nucleic acid as set forth herein with another such nucleic acid, or an additional nucleic acid. Corresponding sequence strings in a database present in a computer system or computer readable medium are a feature of the invention.

[0232] DNA mutagenesis and recursive recombination provide a robust, widely applicable, means of generating diversity useful for the engineering of proteins, pathways, cells and organisms with improved characteristics. In addition to the basic formats described above, it is sometimes desirable to combine recursive recombination methodologies with other techniques for generating diversity. In conjunction with (or separately from) recursive recombination methods, a variety of diversity generation methods can be practiced and the results (i.e., diverse populations of nucleic acids) screened for in the systems of the invention.

Additional diversity can be introduced by methods which result in the alteration of individual nucleotides or groups of contiguous or non-contiguous nucleotides, i.e., mutagenesis methods. Many mutagenesis methods are found in the above-cited references; additional details regarding mutagenesis methods can be found in the references listed below.

[0233] Mutagenesis methods include, for example, recombination (*see:* PCT/US98/05223, WO98/42727); site-directed mutagenesis, mutagenesis using uracil containing templates, oligonucleotide-directed mutagenesis, phosphorothioate-modified DNA mutagenesis, and mutagenesis using gapped duplex DNA. A variety of references for these methods of mutagenesis have been provided *supra.*

[0234] Additional suitable methods include point mismatch repair, mutagenesis using repair-deficient host strains, deletion mutagenesis, restriction-selection and restriction-selection and restriction-purification, mutagenesis by total gene synthesis, and double-strand break repair. A variety of references for these various mutagenesis techniques have been provided *supra.* Furthermore, additional details on many of the above methods can be found in *Methods in Enzymology* Volume 154, which also describes useful controls for trouble-shooting problems with various mutagenesis methods.

[0235] Mutagenesis employing polynucleotide chain termination methods have also been proposed (*see e.g.*, U.S. Patent No. 5,965,408 and other references *supra*), and can be applied to the present invention. In this approach, double stranded DNAs corresponding to one or more genes sharing regions of sequence similarity are combined and denatured, in the presence or absence of primers specific for the gene. The single stranded polynucleotides are then annealed and incubated in the presence of a polymerase and a chain terminating reagent (*e.g.*, ultraviolet, gamma or X-ray irradiation; ethidium bromide or other intercalators; DNA binding proteins, such as single strand binding proteins, transcription activating factors, or histones; polycyclic aromatic hydrocarbons; trivalent chromium or a trivalent chromium salt; or abbreviated polymerization mediated by rapid thermocycling; and the like), resulting in the production of partial duplex molecules. The partial duplex molecules, *e.g.*, containing partially extended chains, are then denatured and re-annealed in subsequent rounds of replication or partial replication resulting in polynucleotides which share varying degrees of sequence similarity and which are diversified with respect to the starting population of DNA molecules. Optionally, the products, or partial pools of the products, can be amplified at one or more stages in the process. Polynucleotides produced by a chain termination method, such as described above, are suitable substrates for any other described recombination format.

[0236] Diversity also can be generated in nucleic acids or populations of nucleic acids using a recombinational procedure termed "incremental truncation for the creation of hybrid enzymes" ("ITCHY") described in Ostermeier *et al.* (1999) *Nat Biotech* 17:1205. This approach can be used to generate an initial library of variants which can optionally serve as a substrate for one or more in vitro or *in vivo* recombination methods. See, also, Ostermeier *et al.* (1999) *PNAS USA*, 96: 3562-67; Ostermeier *et al.* (1999) *Biological and Medicinal Chemistry*, 7: 2139-44.

[0237] Mutational methods which result in the alteration of individual nucleotides or groups of contiguous or non-contiguous nucleotides can be favorably employed to introduce nucleotide diversity, i.e., to introduce nucleotide diversity into FD/FDD homologues, etc. Many mutagenesis methods are found in the above-cited references; additional details regarding mutagenesis methods can be found in following, which can also be applied to the present invention.

[0238] For example, error-prone PCR can be used to generate nucleic acid variants. Using this technique, PCR is performed under conditions where the copying fidelity of the DNA polymerase is low, such that a high rate of point mutations is obtained along the entire length of the PCR product. Examples of such techniques are found in the references above and, *e.g.*, in Leung *et al.* (1989) *Technique* 1:11-15 and Caldwell *et al.* (1992) *PCR Methods Applic* 2:28-33. Similarly, assembly PCR can be used, in a process which involves the assembly of a PCR product from a mixture of small DNA fragments. A large number of different PCR reactions can occur in parallel in the same reaction mixture, with the products of one reaction priming the products of another reaction.

[0239] Sexual PCR mutagenesis can be used in which homologous recombination occurs between DNA molecules of different but related DNA sequence in vitro, by random fragmentation of the DNA molecule based on sequence homology, followed by fixation of the crossover by primer extension in a PCR reaction. This process is described in the references above, *e.g.*, in Stemmer (1994) *PNAS USA* 91:10747-10751. Recursive ensemble mutagenesis can be used in which an algorithm for protein mutagenesis is used to produce diverse populations of phenotypically related mutants whose members differ in amino acid sequence. This method uses a feedback mechanism to control successive rounds of combinatorial cassette mutagenesis. Examples of this approach are found in Arkin & Youvan (1992) *PNAS USA* 89:7811-7815.

[0240] Oligonucleotide directed mutagenesis can be used to introduce site-specific mutations in a nucleic acid sequence of interest. Examples of such techniques are found in

the references above and, *e.g.*, in Reidhaar-Olson *et al.* (1988) *Science*, 241:53-57. Similarly, cassette mutagenesis can be used in a process that replaces a small region of a double stranded DNA molecule with a synthetic oligonucleotide cassette that differs from the native sequence. The oligonucleotide can contain, *e.g.*, completely and/or partially randomized native sequence(s).

[0241] Exponential ensemble mutagenesis can be used for generating combinatorial libraries with a high percentage of unique and functional mutants. Small groups of residues in a sequence of interest are randomized in parallel to identify, at each altered position, amino acids which lead to functional proteins. Examples of such procedures are found in Delegrave & Youvan (1993) *Biotechnology Research* 11:1548-1552.

[0242] In vivo mutagenesis can be used to generate random mutations in any cloned DNA of interest by propagating the DNA, *e.g.*, in a strain of *E. coli* that carries mutations in one or more of the DNA repair pathways. These "mutator" strains have a higher random mutation rate than that of a wild-type parent. Propagating the DNA in one of these strains will eventually generate random mutations within the DNA. Such procedures are described in the references noted above.

[0243] Other procedures for introducing diversity into a genome, *e.g.* a bacterial, fungal, animal or plant genome can be used in conjunction with the above described and/or referenced methods. For example, in addition to the methods above, techniques have been proposed which produce nucleic acid multimers suitable for transformation into a variety of species (*see, e.g.* U.S. Patent No. 5,756,316 and the references *supra*). Transformation of a suitable host with such multimers, consisting of genes that are divergent with respect to one another, (*e.g.*, derived from natural diversity or through application of site directed mutagenesis, error prone PCR, passage through mutagenic bacterial strains, and the like), provides a source of nucleic acid diversity for DNA diversification, *e.g.*, by an *in vivo* recombination process as indicated above.

[0244] Alternatively, a multiplicity of monomeric polynucleotides sharing regions of partial sequence similarity can be transformed into a host species and recombined *in vivo* by the host cell. Subsequent rounds of cell division can be used to generate libraries, members of which include a single, homogenous population, or pool of monomeric polynucleotides. Alternatively, the monomeric nucleic acid can be recovered by standard techniques, *e.g.*, PCR and/or cloning, and recombined in any of the recombination formats, including recursive recombination formats, described above.

[0245] Methods for generating multispecies expression libraries have been described (in addition to the reference noted above, *see, e.g.*, U.S. Pat. No. 5,783,431 and U.S. Pat. No. 5,824,485) and their use to identify protein activities of interest has been proposed (In addition to the references noted above, *see*, U.S. Pat. No. 5,958,672). Multispecies expression libraries include, in general, libraries comprising cDNA or genomic sequences from a plurality of species or strains, operably linked to appropriate regulatory sequences, in an expression cassette. The cDNA and/or genomic sequences are optionally randomly ligated to further enhance diversity. The vector can be a shuttle vector suitable for transformation and expression in more than one species of host organism, *e.g.*, bacterial species, eukaryotic cells. In some cases, the library is biased by preselecting sequences which encode a protein of interest, or which hybridize to a nucleic acid of interest. Any such libraries can be provided as substrates for any of the methods herein described.

[0246] The above described procedures have been largely directed to increasing nucleic acid and/ or encoded protein diversity. However, in many cases, not all of the diversity is useful, *e.g.*, functional, and contributes merely to increasing the background of variants that must be screened or selected to identify the few favorable variants. In some applications, it is desirable to preselect or prescreen libraries (*e.g.*, an amplified library, a genomic library, a cDNA library, a normalized library, etc.) or other substrate nucleic acids prior to diversification, *e.g.*, by recombination-based mutagenesis procedures, or to otherwise bias the substrates towards nucleic acids that encode functional products. For example, in the case of antibody engineering, it is possible to bias the diversity generating process toward antibodies with functional antigen binding sites by taking advantage of *in vivo* recombination events prior to manipulation by any of the described methods. For example, recombined CDRs derived from B cell cDNA libraries can be amplified and assembled into framework regions (*e.g.*, Jirholt *et al.* (1998) *Gene* 215: 471) prior to diversifying according to any of the methods described herein.

[0247] Libraries can be biased towards nucleic acids which encode proteins with desirable enzyme activities. For example, after identifying a clone from a library which exhibits a specified activity (*e.g.*, an FD/FDD homologue that detoxifies a fumonisin, etc.), the clone can be mutagenized using any known method for introducing DNA alterations. A library comprising the mutagenized homologues is then screened for a desired activity, which can be the same as or different from the initially specified activity. An example of such a procedure is proposed in U.S. Patent No. 5,939,250. Desired activities can be identified by any method known in the art. For example, WO 99/10539 proposes that gene libraries can be screened by combining extracts from the gene library with components obtained from

metabolically rich cells and identifying combinations which exhibit the desired activity. It has also been proposed (*e.g.*, WO 98/58085) that clones with desired activities can be identified by inserting bioactive substrates into samples of the library, and detecting bioactive fluorescence corresponding to the product of a desired activity using a fluorescent analyzer, *e.g.*, a flow cytometry device, a CCD, a fluorometer, or a spectrophotometer.

[0248] Libraries can also be biased towards nucleic acids which have specified characteristics, *e.g.*, hybridization to a selected nucleic acid probe. For example, application WO 99/10539 proposes that polynucleotides encoding a desired activity (*e.g.*, an enzymatic activity, for example: a lipase, an esterase, a protease, a glycosidase, a glycosyl transferase, a phosphatase, a kinase, an oxygenase, a peroxidase, a hydrolase, a hydratase, a nitrilase, a transaminase, an amidase or an acylase) can be identified from among genomic DNA sequences in the following manner. Single stranded DNA molecules from a population of genomic DNA are hybridized to a ligand-conjugated probe. The genomic DNA can be derived from either a cultivated or uncultivated microorganism, or from an environmental sample. Alternatively, the genomic DNA can be derived from a multicellular organism, or a tissue derived therefrom. Second strand synthesis can be conducted directly from the hybridization probe used in the capture, with or without prior release from the capture medium or by a wide variety of other strategies known in the art. Alternatively, the isolated single-stranded genomic DNA population can be fragmented without further cloning and used directly in, *e.g.*, a recombination-based approach, that employs a single-stranded template, as described above.

[0249] In one such method the fragment population derived the genomic library(ies) is annealed with partial, or, often approximately full length ssDNA or RNA corresponding to the opposite strand. Assembly of complex chimeric genes from this population is then mediated by nuclease-based removal of non-hybridizing fragment ends, polymerization to fill gaps between such fragments and subsequent single stranded ligation. The parental strand can be removed by digestion (if RNA or uracil-containing), magnetic separation under denaturing conditions (if labeled in a manner conducive to such separation) and other available separation/purification methods. Alternatively, the parental strand is optionally co-purified with the chimeric strands and removed during subsequent screening and processing steps. As set forth in "Single-stranded nucleic acid template-mediated recombination and nucleic acid fragment isolation" by Affholter (USSN 60/186,482, filed March 2,2000), recursive recombination using single-stranded templates and nucleic acids of interest which bind to a portion of the template can also be performed.

[0250] In one approach, single-stranded molecules are converted to double-stranded DNA (dsDNA) and the dsDNA molecules are bound to a solid support by ligand-mediated binding. After separation of unbound DNA, the selected DNA molecules are released from the support and introduced into a suitable host cell to generate a library enriched in sequences which hybridize to the probe. A library produced in this manner provides a desirable substrate for any of the recursive recombination reactions described herein.

[0251] "Non-Stochastic" methods of generating nucleic acids and polypeptides are alleged in Short "Non-Stochastic Generation of Genetic Vaccines and Enzymes" WO 00/46344. These methods, including proposed non-stochastic polynucleotide reassembly and site-saturation mutagenesis methods be applied to the present invention as well. Random or semi-random mutagenesis using doped or degenerate oligonucleotides is also described in, *e.g.*, Arkin and Youvan (1992) *Biotechnology* 10:297-300; Reidhaar-Olson *et al.* (1991) *Methods Enzymol.* 208:564-86; Lim and Sauer (1991) *J Mo. Biol* 219:359-76; Breyer and Sauer (1989) *J Biol Chem* 264:13355-60; and US Patents 5,830,650 and 5,798,208, and EP Patent 0527809 B1.

[0252] It will readily be appreciated that any of the above described techniques suitable for enriching a library prior to diversification can also be used to screen the products, or libraries of products, produced by the diversity generating methods.

[0253] Kits for mutagenesis, library construction and other diversity generation methods are also commercially available. For example, kits are available from, *e.g.*, Stratagene (*e.g.*, QuickChange™ site-directed mutagenesis kit; and Chameleon™ double-stranded, site-directed mutagenesis kit), Bio/Can Scientific, Bio-Rad (*e.g.*, using the Kunkel method described above), Boehringer Mannheim Corp., Clonetech Laboratories, DNA Technologies, Epicentre Technologies (*e.g.*, 5 prime 3 prime kit); Genpak Inc, Lemargo Inc, Life Technologies (Gibco BRL), New England Biolabs, Pharmacia Biotech, Promega Corp., Quantum Biotechnologies, Amersham International plc (*e.g.*, using the Eckstein method above), and Anglian Biotechnology Ltd (*e.g.*, using the Carter/Winter method above).

[0254] The above references provide many mutational formats, including recombination, recursive recombination, recursive mutation and combinations or recombination with other forms of mutagenesis, as well as many modifications of these formats. Regardless of the diversity generation format that is used, the nucleic acids of the invention can be recombined (with each other, or with related (or even unrelated) sequences) to produce a diverse set of recombinant nucleic acids, including, *e.g.*, sets of homologous nucleic acids, as well as corresponding polypeptides.

OTHER POLYNUCLEOTIDE COMPOSITIONS

[0255] The invention also includes compositions comprising any two or more polynucleotides (*e.g.*, 2 or more, 5 or more, or 20, 50, 100 or more, etc.) of the invention (*e.g.*, as substrates for recombination). The composition can comprise a library of recombinant nucleic acids, where the library contains at least 2, at least 3, at least 5, at least 10, at least 20, or at least 50 or more nucleic acids. The nucleic acids are optionally cloned into expression vectors, providing expression libraries. Additionally, in various aspects the invention also includes fragments of polypeptides that have fumonisin and/or fumonisin-derivative detoxification activity.

[0256] The invention also includes compositions produced by digesting one or more polynucleotide of the invention with a restriction endonuclease, an RNAse, or a DNAse (*e.g.*, as is performed in certain of the recombination formats noted above); and compositions produced by fragmenting or shearing one or more polynucleotide of the invention by mechanical means (*e.g.*, sonication, vortexing, and the like), which can also be used to provide substrates for recombination in the methods above. Similarly, compositions comprising sets of oligonucleotides corresponding to more than one nucleic acid of the invention are useful as recombination substrates and are a feature of the invention. For convenience, these fragmented, sheared, or oligonucleotide synthesized mixtures are referred to as fragmented nucleic acid sets.

[0257] Also included in the invention are compositions produced by incubating one or more of the fragmented nucleic acid sets in the presence of ribonucleotide or deoxyribonucleotide triphosphates and a nucleic acid polymerase. This resulting composition forms a recombination mixture for many of the recombination formats noted above. The nucleic acid polymerase may be an RNA polymerase, a DNA polymerase, or an RNA-directed DNA polymerase (*e.g.*, a "reverse transcriptase"); the polymerase can be, *e.g.*, a thermostable DNA polymerase (such as, VENT, TAQ, or the like).

FD/FDD HOMOLOGUE POLYPEPTIDES

[0258] The invention provides isolated or recombinant fumonisin detoxification or fumonisin-derivative detoxification homologue polypeptides, referred to herein as "FD/FDD homologue polypeptides" or "FD/FDD homologues." An isolated or recombinant FD/FDD homologue polypeptide of the invention includes a polypeptide comprising a sequence selected from SEQ ID NOs:26-50, SEQ ID NOs:70-72, and SEQ ID NOs:85, 87, 89, 91, 93,

95, 97, 99, 101, 103, 105, 107, 109, 111, 113, 115, 117, 119, 121, 123, 125, and 127, and conservatively modified variations thereof.

[0259] In some aspects, the invention comprises an isolated or recombinant polypeptide that is at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99%, or at least about 99.5%, or more identical to (or is substantially identical to, or comprises) one or more of SEQ ID NOs:26-50, SEQ ID NOs:70-72, and SEQ ID NOs:85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107, 109, 111, 113, 115, 117, 119, 121, 123, 125, and 127 (*e.g.*, to SEQ ID NO:50) over a comparison window of at least 100, at least 125, at least 150, at least 175, at least 200, at least 225, at least 250, at least 275, or at least 300 contiguous amino acids wherein the polypeptide has a fumonisin detoxification activity or a fumonisin derivative detoxification activity that is at least 1.5x, at least 2x, at least 5x, at least 10x, at least 15x, at least 20x, or at least 25x or more greater than any of the polypeptides corresponding to ESP002C2, ESP002C3, ESP003C12, RAT011C1, RAT011D2, RAT011C4, or wild-type APAO (i.e., as listed in SEQ ID NOs:51-64). Optionally, the above polypeptide displays its increased FD/FDD activity at pH 5.5 or has an optimum pH lower than that for the polypeptides encoded by SEQ ID NOs:51-64. Additionally, the above polypeptide displays a greater thermostability (i.e., a higher thermostability) than that of any of the polypeptides encoded by SEQ ID NOs:51-64 and/or optionally has increased FD/FDD activity upon secretion from a eukaryotic cell (*e.g.*, a plant cell) relative to that activity of any polypeptide encoded by SEQ ID NOs:51-64. In some embodiments, the polypeptide comprises a leader sequence that directs secretion of the polypeptide from a plant cell (*e.g.*, an apoplast targeting sequence, a peroxisomal targeting sequence, etc.), alternately and/or additionally, the polypeptide optionally comprises a polypeptide purification sequence.

[0260] Furthermore, in some embodiments, such above polypeptide is at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99%, or at least about 99.5%, or more identical to, or is substantially identical to, or is chosen from any one or more of SEQ ID NOs:26-50, SEQ ID NOs:70-72, and SEQ ID NOs:85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107, 109, 111, 113, 115, 117, 119, 121, 123, 125, and 127. Such polypeptide is also, in some embodiments encoded by a polynucleotide selected from SEQ ID NOs:1-25, SEQ ID NOs:67-69, and SEQ ID NOs:84, 86, 86, 99, 92, 94, 96, 98, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, 120, 122, 124, and 126. The FD/FDD activity of such polypeptide is, in typical embodiments, the ability to deaminate fumonisin and/or

fumonisin derivatives (*e.g.*, fumonisin B1, fumonisin B2, fumonisin B3, fumonisin B4, fumonisin C1, or a structural analog, etc.), i.e., the polypeptide is a fumonisin amine oxidase. In yet other embodiments, the above polypeptide of the invention displays one or more of: a k_{cat} (optionally at pH 5.5) greater than, or higher than the k_{cat} of any of the polypeptides encoded by SEQ ID NOs:51-64; a K_M value (optionally at pH 5.5) lower than the K_M value of any of the polypeptides encoded by SEQ ID NOs:51-64; or a k_{cat}/K_M value higher than, or greater than the k_{cat}/K_M value of any of the polypeptides encoded by SEQ ID NOs:51-64 when catalyzing a fumonisin or fumonisin-detoxification reaction (*e.g.*, a fumonisin deamination reaction).

[0261] In some optional embodiments of the invention, the above polypeptide comprises variants wherein one or more amino acid has been mutated. In yet other embodiments, the above polypeptide comprises an alanine residue at position 118, a serine residue at position 136, a phenylalanine reside at position 209, a lysine residue at position 210, an isoleucine residue at position 237, a glutamic acid residue at position 272, a proline residue at position 274, and a glutamic acid residue at position 473, wherein the recited positions refer to amino acid positions in the polypeptide of the wild type APAO enzyme (SEQ ID NO: 52). In yet other embodiments, the above polypeptide comprises an aspartic acid residue at position 193.

[0262] In some embodiments, the alteration of residue 193 can optionally change enzymatic performance of the polypeptide (*e.g.*, k_{cat} of a fumonisin detoxification reaction, such as fumonisin deamination, catalyzed by the polypeptide can be changed). For example, an aspartic acid at residue 193 (as opposed to an asparagine at residue 193, as is found in wild-type APAO, see SEQ ID NO: 52) optionally leads to an increased k_{cat} of the fumonisin detoxification (deamination) reaction. Some embodiments of the current invention also comprise polypeptides with an altered glycosylation site.

Methods of use of FD/FDD Polypeptides

[0263] The polypeptides of the current invention are useful in numerous ways. For example, a method of detoxifying, degrading, neutralizing, deaminating, or modifying at least one mycotoxin or mycotoxin derivative through incubation of such mycotoxin with at least one polypeptide of the invention (as described herein) where such polypeptide detoxifies, degrades, neutralizes, deaminates, or modifies the mycotoxin/mycotoxin-derivative is an optional feature of the invention. This optionally includes wherein the mycotoxin is a fumonisin, a fumonisin-derivative and/or a fumonisin analogue and wherein such mycotoxin

is present in harvested foodstuffs (*e.g.*, grain), unharvested foodstuffs (*e.g.*, crops/plants in field, etc.), silage, etc. and also wherein the degradation/detoxification occurs during harvesting, processing, or storage, of the material.

[0264] Other features of the invention illustrating (non-limiting) uses of the polynucleotides/polypeptides of the invention can be found *sic passim* (*see*, *e.g.*, *In Vivo* Uses, *supra*).

[0265] Further aspects of the invention include a method of reducing pathogenicity of a fungus producing fumonisin comprising: a) providing a transgenic cell with any nucleic acid of the invention operably linked to a promoter wherein the nucleic acid is heterologous to the cell, and b) expressing the nucleic acid at a level effective to detoxify the fumonisin, thereby reducing the pathogenicity of the fungus. Such method also optionally comprises wherein the cell is a plant cell in a plant and wherein the cell is a microorganism and wherein the cell comprises a fumonisin esterase encoding polynucleotide operably linked to a promoter.

[0266] Another feature of the invention includes a method of detecting fumonisins comprising: a) introducing any polypeptide of the invention into a sample containing fumonisin, b) allowing the polypeptide to catalyze the deamination of fumonisin, and c) detecting a product of the deamination reaction. Such methods are especially useful in detecting contamination of foodstuffs by fumonisin producing fungi (*e.g.*, *Fusarium moniliforme* or *F. proliferatum*), since, as detailed above, such contamination can have severe health consequences to animals and humans who consume contaminated products. Additionally since mycotoxin contaminated products are monitored/controlled, discovery of contamination can present monetary savings as well (*e.g.*, detection of contaminated corn prevents purchase of such.)

[0267] The FD/FDD polypeptides of the current invention are also optionally used in conjunction with other enzymes to help in detoxification/degradation/etc. of mycotoxins (*e.g.*, fumonisin, etc.). For example, fumonisin esterase, which reduces, but does not eliminate the toxicity of fumonisin can optionally be used in combination with the FD/FDD polypeptides of the invention. The fumonisin esterase converts, *e.g.*, FB1 into AP1, which is also a target for the deamination action of the FD/FDD polypeptides of the invention. Such optional combinations of enzymes (*e.g.*, FD/FDD enzymes and fumonisin esterase) can be co-expressed in an expression system or they can be expressed separately and applied sequentially or in combination to such things as grains, crops, etc.

[0268] Another feature of the invention comprises a method of use, as described *supra*, wherein the FD/FDD polypeptides of the invention are used to decontaminate foodstuffs (*e.g.*, grain) prior to use/consumption of such. The decontamination optionally occurs during the processing of the foodstuff, during processing of a plant material for silage, or during the growth of a crop (*e.g.*, while the crop/plant is still in field, etc.). Such methods optionally comprise presenting the FD/FDD enzymes to the foodstuff/plant/etc. at an appropriate stage in the process/growth cycle/harvest period and in an amount effective to achieve the desired goal (i.e., in an amount effective to reduce and/or eliminate the contamination).

[0269] Yet other methods featured in the current invention comprise treatment of foodstuffs/silage/crop plants with microorganisms which comprise the FD/FDD homologues of the invention and which optionally express the same. For example, various bacteria, yeasts, etc. are capable of being engineered with the FD/FDD homologues of the invention and then inducibly and/or constitutively expressing such polypeptides. These microorganisms are optionally sprayed or inoculated (here meaning deposition of a microorganism which will multiply on, or within, the plant/seed/etc.) onto crops/plants/seeds/etc. where they optionally express (and optionally secrete extracellularly), the FD/FDD homologues of the invention, thereby detoxifying fumonisin on/in the plant or seed, etc. The microorganisms used can optionally be deposited in a suspended liquid form, a lyophilized dust form, or any other convenient manner to effectively coat/inoculate the plant/seed with the FD/FDD expressing microorganism at the appropriate time. A number of suitable microorganisms are known to those skilled in the art, and selection of the appropriate microorganism will vary depending upon, *e.g.*, the type of plant/seed to be protected, the environmental conditions present, etc.

[0270] Additionally, the current invention comprises methods for production of ruminant microorganisms which contain and optionally express the FD/FDD polypeptides of the invention. Such microorganisms are optionally inoculated into, *e.g.*, silage, etc. to act as a ruminant inoculate so as to protect animals from fumonisin poisoning.

[0271] Another feature of the invention comprises methods of use of the FD/FDD polypeptides of the invention in detection of fumonisins and fumonisin-derivatives/analogs. For example, putatively contaminated grain can optionally be tested and any amount of contamination optionally quantified through use of the FD/FDD polypeptides herein. Through determination and measurement of the end products, etc. from fumonisin degradation (*see*, description of assays, *infra*) the amount of fumonisin contamination in a sample can be determined.

Making Polypeptides

[0272] Recombinant methods for producing and isolating FD/FDD homologue polypeptides of the invention are described above. In addition to recombinant production, the polypeptides may be produced by direct peptide synthesis using solid-phase techniques (*see, e.g.*, Stewart *et al.* (1969) *Solid-Phase Peptide Synthesis*, WH Freeman Co, San Francisco; Merrifield J (1963) *J Am Chem Soc* 85:2149-2154). Peptide synthesis may be performed using manual techniques or by automation. Automated synthesis may be achieved, for example, using Applied Biosystems 431A Peptide Synthesizer (Perkin Elmer, Foster City, Calif.) in accordance with the instructions provided by the manufacturer. For example, subsequences may be chemically synthesized separately and combined using chemical methods to provide full-length FD/FDD homologues or fragments thereof. Alternately, such sequences may be ordered from any number of companies which specialize in production of polypeptides. Most commonly FD/FDD polypeptides are produced by expressing coding nucleic acids and recovering polypeptides, *e.g.*, as described above. For example, one feature of the current invention is a method of producing a polypeptide through: a) introducing into a population of cells (*e.g.*, plant cells, yeasts, etc.) any nucleic acid of the invention as described herein (*e.g.*, any of SEQ ID NOs:1-25, SEQ ID NOs:67-69, and SEQ ID NOs:84, 86, 86, 99, 92, 94, 96, 98, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, 120, 122, 124, and 126 or fragments/modifications/complements thereof) which is operably linked to a regulatory sequence effective to produce the encoded polypeptide, b) culturing the cells in a culture medium to produce the polypeptide, and c) isolating the polypeptide from the cells or culture medium.

Using Polypeptides

Antibodies

[0273] In another aspect of the invention, an FD/FDD homologue polypeptide of the invention is used to produce antibodies which have, *e.g.*, diagnostic uses, *e.g.*, related to the activity, distribution, and expression of FD/FDD homologues, *e.g.* in various tissues of a transgenic plant.

[0274] Other optional embodiments of the invention comprise polypeptides that are specifically bound by polyclonal antisera raised against one or more antigen from SEQ ID NOs:26-50, SEQ ID NOs:70-72, and SEQ ID NOs:85, 87, 89, 91, 93, 95, 97, 99, 101, 103,

105, 107, 109, 111, 113, 115, 117, 119, 121, 123, 125, and 127 (or a fragment thereof), wherein the antisera is subtracted with one of more polypeptide from SEQ ID NOs:51-64. Additionally, the invention also optionally includes polypeptides which comprise a unique subsequence in a polypeptide selected from SEQ ID NOs:26-50, SEQ ID NOs:70-72, and SEQ ID NOs:85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107, 109, 111, 113, 115, 117, 119, 121, 123, 125, and 127, wherein the subsequence is unique as compared to a polypeptide corresponding to any of SEQ ID NOs:51-64.

[0275] Antibodies to FD/FDD homologues of the invention may be generated by methods well known in the art. Such antibodies may include, but are not limited to, polyclonal, monoclonal, chimeric, humanized, single chain, Fab fragments and fragments produced by a Fab expression library.

[0276] FD/FDD homologue polypeptides for antibody induction do not require biological activity; however, the polypeptide or oligopeptide are antigenic. Peptides used to induce specific antibodies may have an amino acid sequence consisting of at least 10 amino acids, preferably at least 15 or 20 amino acids. Short stretches of an FD/FDD homologue polypeptide may be fused with another protein, such as keyhole limpet hemocyanin, and antibody produced against the chimeric molecule.

[0277] Methods of producing polyclonal and monoclonal antibodies are known to those of skill in the art, and many antibodies are available. *See, e.g.*, Coligan (1991) *Current Protocols in Immunology* Wiley/Greene, NY; and Harlow and Lane (1989) *Antibodies: A Laboratory Manual*, Cold Spring Harbor Press, NY (hereinafter "Harlow and Lane"); Stites *et al.* (eds.) *Basic and Clinical Immunology* (4th ed.) Lange Medical Publications, Los Altos, CA, and references cited therein; Goding (1986) *Monoclonal Antibodies: Principles and Practice* (2d ed.) Academic Press, New York, NY; and Kohler and Milstein (1975) *Nature* 256:495-497. Other suitable techniques for antibody preparation include selection of libraries of recombinant antibodies in phage or similar vectors. *See*, Huse *et al.* (1989) *Science* 246:1275-1281; and Ward *et al.* (1989) *Nature* 341:544-546. Specific monoclonal and polyclonal antibodies and antisera will usually bind with a K_D of at least about 0.1 μM, preferably at least about 0.01 μM or better, and most typically and preferably, 0.001 μM or better.

[0278] Additional details about antibody production and engineering techniques can be found in Borrebaeck (ed.) (1995) *Antibody Engineering*, 2nd Edition, Freeman and Company, NY; McCafferty *et al.* (1996) *Antibody Engineering, A Practical Approach* IRL at

Oxford Press, Oxford, England; and Paul (1995) *Antibody Engineering Protocols* Humana Press, Towata, NJ.

DEFINING POLYPEPTIDES BY IMMUNOREACTIVITY

[0279] Because the polypeptides of the invention provide a variety of new polypeptide sequences as compared to other FD/FDD homologues, the polypeptides also provide new structural features which can be recognized, *e.g.*, in immunological assays. The generation of antisera which specifically binds the polypeptides of the invention, as well as the polypeptides which are bound by such antisera, are a feature of the invention.

[0280] The invention includes FD/FDD homologue proteins that specifically bind to or that are specifically immunoreactive with an antibody or antisera generated against an immunogen comprising an amino acid sequence selected from one or more of SEQ ID NOs:26-50, SEQ ID NOs:70-72, and SEQ ID NOs:85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107, 109, 111, 113, 115, 117, 119, 121, 123, 125, and 127. To eliminate cross-reactivity with other polypeptides, the cross-reactive antibody or antisera is removed from the antisera by, for example, immunosorption with polypeptides encoded by sequences such as those represented by clone numbers ESP002C2, ESP002C3, ESP003C12, RAT011C1, RAT011C2, RAT011C4 from PCT publications WO 00/04159 and WO 00/04160 or wild-type APAO (SEQ ID NO:52) or by similar homologous mycotoxin detoxifying molecules found in, *e.g.*, GenBank (the polypeptides). Where the accession number corresponds to a nucleic acid, a polypeptide encoded by the nucleic acid is generated and used for antibody/antisera subtraction purposes. Where the nucleic acid corresponds to a non-coding sequence, *e.g.*, a pseudo gene, an amino acid which corresponds to the reading frame of the nucleic acid is generated (*e.g.*, synthetically), or is minimally modified to include a start codon, promoter or the like for recombinant production.

[0281] In one typical format, the immunoassay uses a polyclonal antiserum which was raised against one or more polypeptide comprising one or more of the sequences corresponding to one or more of: SEQ ID NOs:26-50, SEQ ID NOs:70-72, and SEQ ID NOs:85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107, 109, 111, 113, 115, 117, 119, 121, 123, 125, and 127, or a substantial subsequence thereof (i.e., at least about 30% of the full length sequence provided). The full set of potential polypeptide immunogens derived from SEQ ID NOs:26-50, SEQ ID NOs:70-72, and SEQ ID NOs:85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107, 109, 111, 113, 115, 117, 119, 121, 123, 125, and 127 are collectively referred to below as "the immunogenic polypeptides." The resulting antisera is optionally selected to have low cross-reactivity against the control polypeptides (*e.g.*, ESP002C2,

ESP002C3, ESP003C12, RAT011C1, RAT011C2, RAT011C4 and wild type APOA) and any other known related polypeptides and any such cross-reactivity is removed by immunoabsorbtion with one or more of the control polypeptides, prior to use of the polyclonal antiserum in the immunoassay. Sequences which are substantially identical to such sequences can also be used, *e.g.*, which are about 80%, about 90%, about 95%, about 98%, about 99%, about 99.5% or more identical, *e.g.*, as determined using BLAST or the other algorithms described above, *e.g.*, using default parameters.

[0282] In order to produce antisera for use in an immunoassay, one or more of the immunogenic polypeptides is produced and purified as described herein. For example, recombinant protein may be produced in a bacterial cell line. An inbred strain of mice (used in this assay because results are more reproducible due to the virtual genetic identity of the mice) is immunized with the immunogenic protein(s) in combination with a standard adjuvant, such as Freund's adjuvant, and a standard mouse immunization protocol (*see*, Harlow and Lane for a standard description of antibody generation, immunoassay formats and conditions that can be used to determine specific immunoreactivity). Alternatively, one or more synthetic or recombinant polypeptide derived from the sequences disclosed herein is conjugated to a carrier protein and used as an immunogen.

[0283] Polyclonal sera are collected and titered against the immunogenic polypeptide in an immunoassay, for example, a solid phase immunoassay with one or more of the immunogenic proteins immobilized on a solid support. Polyclonal antisera with a titer of 10^6 or greater are selected, pooled and subtracted with the control polypeptides, *e.g.* those identified from, *e.g.*, GenBank or elsewhere as per above, to produce subtracted pooled titered polyclonal antisera.

[0284] The subtracted, pooled, titered polyclonal antisera are tested for cross reactivity against the control polypeptides. Preferably at least two of the immunogenic FD/FDD homologues are used in this determination, preferably in conjunction with at least two of the control polypeptides, to identify antibodies which are specifically bound by the immunogenic protein(s).

[0285] In this comparative assay, discriminatory binding conditions are determined for the subtracted, titered polyclonal antisera which result in at least about a 5-10 fold higher signal to noise ratio for binding of the titered polyclonal antisera to the immunogenic FD/FDD homologues as compared to binding to any of the control polypeptides. That is, the stringency of the binding reaction is adjusted by the addition of non-specific competitors such as albumin or non-fat dry milk, or by adjusting salt conditions, temperature, or the like.

These binding conditions are used in subsequent assays for determining whether a test polypeptide is specifically bound by the pooled, subtracted polyclonal antisera. In particular, test polypeptides which show at least a 2-5x higher signal to noise ratio than the control polypeptides under discriminatory binding conditions, and at least about a one-half signal to noise ratio as compared to the immunogenic polypeptide(s), share substantial structural similarity with the immunogenic polypeptide as compared to known FD/FDD polypeptides, and are, therefore polypeptides of the invention.

[0286] In another example, immunoassays in the competitive binding format are used for detection of a test polypeptide. For example, as noted, cross-reacting antibodies are removed from the pooled antisera mixture by immunoabsorbtion with control polypeptides. The immunogenic polypeptide(s) are then immobilized to a solid support which is exposed to the subtracted, pooled antisera. Test proteins are added to the assay to compete for binding to the pooled, subtracted antisera. The ability of the test protein(s) to compete for binding to the pooled, subtracted antisera as compared to the immobilized protein(s) is compared to the ability of the immunogenic polypeptide(s) added to the assay to compete for binding (the immunogenic polypeptides compete effectively with the immobilized immunogenic polypeptides for binding to the pooled antisera). The percent cross-reactivity for the test proteins is calculated, using standard calculations.

[0287] In a parallel assay, the ability of the control proteins to compete for binding to the pooled, subtracted antisera is determined as compared to the ability of the immunogenic polypeptide(s) to compete for binding to the antisera. Again, the percent cross-reactivity for the control polypeptides is calculated, using standard calculations. Where the percent cross-reactivity is at least 5-10x as high for the test polypeptides, the test polypeptides are said to specifically bind the pooled, subtracted antisera.

[0288] In general, the immunoabsorbed and pooled antisera can be used in a competitive binding immunoassay as described herein to compare any test polypeptide to the immunogenic polypeptide(s). In order to make this comparison, the two polypeptides are each assayed at a wide range of concentrations and the amount of each polypeptide required to inhibit 50% of the binding of the subtracted antisera to the immobilized protein is determined using standard techniques. If the amount of the test polypeptide required is less than twice the amount of the immunogenic polypeptide that is required, then the test polypeptide is said to specifically bind to an antibody generated to the immunogenic protein, provided the amount is at least about 5-10x as high as for a control polypeptide.

[0289] As a final determination of specificity, the pooled antisera is optionally fully immunosorbed with the *immunogenic* polypeptide(s) (rather than the control polypeptides) until little or no binding of the resulting immunogenic polypeptide subtracted, pooled antisera to the immunogenic polypeptide(s) used in the immunosorbtion is detectable. This fully immunosorbed antisera is then tested for reactivity with the test polypeptide. If little or no reactivity is observed (i.e., no more than 2x the signal to noise ratio observed for binding of the fully immunosorbed antisera to the immunogenic polypeptide), then the test polypeptide is specifically bound by the antisera elicited by the immunogenic protein.

DETOXIFICATION PROPERTIES OF FD/FDD HOMOLOGUES

Assays for Fumonisin Inactivation

[0290] Screening for the presence of fumonisin detoxification or fumonisin-derivative detoxification capability can be done in a number of ways.

[0291] It is possible to directly select the clones expressing an FD/FDD protein by using, *e.g.*, a yeast strain, if the yeast is susceptible to the compound. For example, Kimura et al (1997) *J Biol Chem* 273(3):1654-1661, describe expression of a mycotoxin detoxifying gene in yeast and selection of the yeast containing such gene in medium containing a potent mycotoxin. This same assay format can be used for any mycotoxin or mycotoxin-derivative which is toxic to yeast, or inhibitory to yeast growth on a medium (i.e., fumonisin or fumonisin-derivative). Similarly, such assays can be performed using any of a variety of other cultured cells, by growing the cells (*e.g.*, prokaryotic or eukaryotic cells) in the presence of a mycotoxin (i.e., fumonisin). Additionally, cells or organisms can be cultured or grown on media wherein, *e.g.*, FB1 is the sole nitrogen source. Thus, only cells or organisms capable of utilizing, *e.g.*, FB1 are able to grow.

[0292] In general, the culture of cells, including yeast, animal cells, plant cells and the like are well known and are discussed in detail *supra* and in references *supra*. It will be appreciated that it is desirable to transform plant cells with fumonisin or fumonisin-derivative resistant nucleic acids in order to reduce food contamination by such mycotoxins and their derivatives and to improve plant resistance to such mycotoxins and their derivatives, *e.g.*, to enhance yield. Accordingly, it can be convenient to screen for fumonisin detoxification or fumonisin-derivative detoxification using plant cells in culture which correspond to the plant which is desired to be transformed.

[0293] If the oxidized products of the detoxification reaction are fluorescent, clones having fumonisin detoxification or fumonisin-detoxification activity are detected by

fluorescence of specific molecules resulting from the detoxification. The intensity of fluorescence may help select clones having higher activity (or higher expression). Example 1 illustrates the use of fluorescence to monitor FD/FDD activity using horse radish peroxidase and Amplex Red. *See, infra.*

[0294] Clones expressing the FD/FDD nucleic acids of the invention can be examined for detoxification activity against one or more than one mycotoxin (i.e., fumonisin) in pools of 10, in order to prescreen the initial transformants rapidly. Any pools showing significant activity can be deconvoluted to identify single desirable clones with high activity and/or broad specificity.

[0295] Some types of FD/FDD activity can be monitored by HPLC, gas chromatography and mass spectroscopy (MS), as well as a variety of other analytical methods available to one of skill. Incorporation of radio-labeled molecules can be monitored directly by mass shift by MS methods and by an appropriate radioisotope detector with HPLC and GC devices. In a high throughput modality, a method of choice is high throughput MS, or MS with an electron spray-based detection method.

[0296] In addition, formation of by-products or end-products of fumonisin detoxification or fumonisin-derivative detoxification can be indirectly measured by various reactive colorimetric reactions through the use of a number of commercially available reactive dyes.

[0297] As is apparent from the foregoing, the relevant assay will depend on the application. Many assay formats are suitable for many applications. Advantageously, any of the assays can be practiced in a high-throughput format.

[0298] In high throughput assays, it is possible to screen up to several thousand different FD/FDD variants in a single day. For example, each well of a microtiter plate can be used to run a separate assay, or, if concentration or incubation time effects are to be observed, every 5-10 wells can test a single variant. Thus, a single standard microtiter plate can assay about 100 (*e.g.*, 96) FD/FDD reactions. If 1536 well plates are used, then a single plate can easily accommodate from about 100 to about 1500 different reactions; it is possible to assay several different plates per day. Assay screens for up to about 6,000-20,000 different assays, (i.e., involving different nucleic acids, encoded proteins, concentrations, etc.) can also be used. Microfluidic approaches to reagent manipulation also have been developed, *e.g.*, by Caliper Technologies (Mountain View, CA).

[0299] In addition to fluidic approaches, it is possible, as mentioned above, simply to grow cells on plates of agar which contain fumonisins or fumonisin-derivatives. Cells which have FD/FDD activity (i.e., due to transformation with FD/FDD nucleic acids of the invention) are able to grow on the plates. This approach offers a simple and high-throughput screening method.

[0300] The ability to detect a subtle increase in the performance of an FD/FDD sequence over that of a parent strain relies on the sensitivity of the assay. The chance of finding the organisms having an improvement in FD/FDD activity is increased by the number of individual mutants that can be screened by the assay. To increase the chances of identifying a pool of sufficient size, a prescreen that increases the number of mutants processed by 10-fold can be used. The goal of the primary screen will be to quickly identify mutants having equal or better product titers than the parent strain(s) and to move only these mutants forward to liquid cell culture for subsequent analysis.

[0301] A number of well known robotic systems have also been developed for solution phase chemistries useful in assay systems. These systems include automated workstations like the automated synthesis apparatus developed by Takeda Chemical Industries, LTD. (Osaka, Japan) and many robotic systems utilizing robotic arms (Zymate II, Zymark Corporation, Hopkinton, MA.; Orca, Hewlett-Packard, Palo Alto, CA) which mimic the manual synthetic operations performed by a scientist. Any of the above devices are suitable for use with the FD/FDD homologues of the present invention. The nature and implementation of modifications to these devices (if any) so that they can operate as discussed herein with reference to the integrated system will be apparent to persons skilled in the relevant art.

[0302] High throughput screening systems are commercially available (*see*, *e.g.*, Zymark Corp., Hopkinton, MA; Air Technical Industries, Mentor, OH; Beckman Instruments, Inc. Fullerton, CA; Precision Systems, Inc., Natick, MA, etc.). These systems typically automate entire procedures including all sample and reagent pipetting, liquid dispensing, timed incubations, and final readings of the microplate in detector(s) appropriate for the assay. These configurable systems provide high throughput and rapid start up as well as a high degree of flexibility and customization.

[0303] The manufacturers of such systems provide detailed protocols for the various high throughput devices. Thus, for example, Zymark Corp. provides technical bulletins describing screening systems for detecting the modulation of gene transcription, ligand

binding, and the like. Microfluidic approaches to reagent manipulation have also been developed, *e.g.*, by Caliper Technologies (Mountain View, CA).

[0304] Optical images viewed (and, optionally, recorded) by a camera or other recording device (*e.g.*, a photodiode and data storage device) are optionally further processed in any of the embodiments herein, *e.g.*, by digitizing the image and/or storing and analyzing the image on a computer. As noted above, in some applications, if FD/FDD products are fluorescent, then optical detection approaches can be appropriate. A variety of commercially available peripheral equipment and software is available for digitizing, storing and analyzing a digitized video or digitized optical image, *e.g.*, using PC (Intel x86 or pentium chip compatible DOS™, OS™ WINDOWS™, WINDOWS NT™ or WINDOWS 95™ based machines), MACINTOSH™, or UNIX based (*e.g.*, SUN™ work station) computers.

[0305] One conventional system carries light from the assay device to a cooled charge-coupled device (CCD) camera, a common use in the art. A CCD camera includes an array of picture elements (pixels). The light from the specimen is imaged on the CCD. Particular pixels corresponding to regions of the specimen (*e.g.*, individual hybridization sites on an array of biological polymers) are sampled to obtain light intensity readings for each position. Multiple pixels are processed in parallel to increase speed. The apparatus and methods of the invention are easily used for viewing any sample, *e.g.* by fluorescent or dark field microscopic techniques.

[0306] Integrated systems for analysis in the present invention typically include a digital computer with high-throughput liquid control software, image analysis software, data interpretation software, a robotic liquid control armature for transferring solutions from a source to a destination operably linked to the digital computer, an input device (*e.g.*, a computer keyboard) for entering data to the digital computer to control high throughput liquid transfer by the robotic liquid control armature and, optionally, an image scanner for digitizing label signals from labeled assay components. The image scanner interfaces with the image analysis software to provide a measurement of optical intensity. Typically, the intensity measurement is interpreted by the data interpretation software to show whether the FD/FDD products are produced.

[0307] In one set of assays, the relative toxicity of fumonisin products produced by modification of FD/FDD enzymes is determined. In particular, toxicity can be evaluated in any of the usual assays for fumonisin toxicity and, optionally, compared to the toxicity of the unmodified fumonisin. In the event that toxicity is reduced, secondary toxic effects of detoxification products can be evaluated using the usual assays for fumonisin activity, or

using additional assays such as cell survival assays, *e.g.*, in the presence of increasing levels of the secondary product. This secondary assay helps to determine which FD/FDD activities are most desirable, i.e., using secondary toxicities of fumonisin or fumonisin-derivative metabolites as a measure of unwanted toxicity.

INTEGRATED SYSTEMS

[0308] The present invention provides computers, computer readable media and integrated systems comprising character strings corresponding to the sequence information herein for the polypeptides and nucleic acids herein, including, *e.g.*, those sequences listed herein and the various silent substitutions and conservative substitutions thereof.

[0309] Various methods and genetic algorithms (GOs) known in the art can be used to detect homology or similarity between different character strings, or can be used to perform other desirable functions such as to control output files, provide the basis for making presentations of information including the sequences and the like. Examples include BLAST, discussed *supra*.

[0310] Thus, different types of homology and similarity of various stringency and length can be detected and recognized in the integrated systems herein. For example, many homology determination methods have been designed for comparative analysis of sequences of biopolymers, for spell-checking in word processing, and for data retrieval from various databases. With an understanding of double-helix pair-wise complement interactions among 4 principal nucleobases in natural polynucleotides, models that simulate annealing of complementary homologous polynucleotide strings can also be used as a foundation of sequence alignment or other operations typically performed on the character strings corresponding to the sequences herein (*e.g.*, word-processing manipulations, construction of figures comprising sequence or subsequence character strings, output tables, etc.). An example of a software package with GOs for calculating sequence similarity is BLAST, which can be adapted to the present invention by inputting character strings corresponding to the sequences herein.

[0311] Similarly, standard desktop applications such as word processing software (*e.g.*, Microsoft Word™ or Corel WordPerfect™) and database software (*e.g.*, spreadsheet software such as Microsoft Excel™, Corel Quattro Pro™, or database programs such as Microsoft Access™ or Paradox™) can be adapted to the present invention by inputting a character string corresponding to the FD/FDD homologues of the invention (either nucleic acids or proteins, or both). For example, the integrated systems can include the foregoing

software having the appropriate character string information, *e.g.*, used in conjunction with a user interface (*e.g.*, a GUI in a standard operating system such as a Windows, Macintosh or LINUX system) to manipulate strings of characters. As noted, specialized alignment programs such as BLAST can also be incorporated into the systems of the invention for alignment of nucleic acids or proteins (or corresponding character strings).

[0312] Integrated systems for analysis in the present invention typically include a digital computer with GO software for aligning sequences, as well as data sets entered into the software system comprising any of the sequences herein. The computer can be, *e.g.*, a PC (Intel x86 or Pentium chip- compatible DOS™, OS2™ WINDOWS™ WINDOWS NT™, WINDOWS95™, WINDOWS98™ LINUX based machine, a MACINTOSH™, Power PC, or a UNIX based (*e.g.*, SUN™ work station) machine) or other commercially common computer which is known to one of skill. Software for aligning or otherwise manipulating sequences is available, or can easily be constructed by one of skill using a standard programming language such as Visualbasic, Fortran, Basic, Java, or the like.

[0313] Any controller or computer optionally includes a monitor which is often a cathode ray tube ("CRT") display, a flat panel display (*e.g.*, active matrix liquid crystal display, liquid crystal display), or others. Computer circuitry is often placed in a box which includes numerous integrated circuit chips, such as a microprocessor, memory, interface circuits, and others. The box also optionally includes a hard disk drive, a floppy disk drive, a high capacity removable drive such as a writeable CD-ROM, and other common peripheral elements. Inputting devices such as a keyboard or mouse optionally provide for input from a user and for user selection of sequences to be compared or otherwise manipulated in the relevant computer system.

[0314] The computer typically includes appropriate software for receiving user instructions, either in the form of user input into a set parameter fields, *e.g.*, in a GUI, or in the form of preprogrammed instructions, *e.g.*, preprogrammed for a variety of different specific operations. The software then converts these instructions to appropriate language for instructing the operation of the fluid direction and transport controller to carry out the desired operation.

[0315] The software can also include output elements for controlling nucleic acid synthesis (*e.g.*, based upon a sequence or an alignment of a sequence herein) or other operations which occur downstream from an alignment or other operation performed using a character string corresponding to a sequence herein.

[0316] In an additional aspect, the present invention provides kits embodying the methods, compositions, systems and apparatus herein. Kits of the invention optionally comprise one or more of the following: (1) an apparatus, system, system component or apparatus component as described herein; (2) instructions for practicing the methods described herein, and/or for operating the apparatus or apparatus components herein and/or for using the compositions herein; (3) one or more FD/FDD composition or component; (4) a container for holding components or compositions, and, (5) packaging materials.

[0317] In a further aspect, the present invention provides for the use of any apparatus, apparatus component, composition or kit herein, for the practice of any method or assay herein, and/or for the use of any apparatus or kit to practice any assay or method herein.

[0318] Below is a table of the clones and sequence identifiers for the polynucleotide and polypeptide sequences of the instant invention.

Table 3: Clones and Sequence Identifiers for the Sequences of the Invention

SEQ ID NO:	Clone ID	SEQ ID NO:	Clone ID
SEQ ID NO:1	A5	SEQ ID NO:26	A5
SEQ ID NO:2	D5	SEQ ID NO:27	D5
SEQ ID NO:3	F7	SEQ ID NO:28	F7
SEQ ID NO:4	F12	SEQ ID NO:29	F12
SEQ ID NO:5	G11	SEQ ID NO:30	G11
SEQ ID NO:6	R3H1	SEQ ID NO:31	R3H1
SEQ ID NO:7	3B12	SEQ ID NO:32	3B12
SEQ ID NO:8	4F13G12	SEQ ID NO:33	4F13G12
SEQ ID NO:9	4F15A11	SEQ ID NO:34	4F15A11
SEQ ID NO:10	4F15C3	SEQ ID NO:35	4F15C3
SEQ ID NO:11	4F16C6	SEQ ID NO:36	4F16C6
SEQ ID NO:12	4F19F2	SEQ ID NO:37	4F16F2
SEQ ID NO:13	4F21C8	SEQ ID NO:38	4F21C8
SEQ ID	4F22B2	SEQ ID	4F22B2

NO:14		NO:39	
SEQ ID NO:15	4F24F2	SEQ ID NO:40	4F24F2
SEQ ID NO:16	4F28G1	SEQ ID NO:41	4F28G1
SEQ ID NO:17	4F2G10	SEQ ID NO:42	4F2G10
SEQ ID NO:18	4F3B5	SEQ ID NO:43	4F3B5
SEQ ID NO:19	4F6A11	SEQ ID NO:44	4F6A11
SEQ ID NO:20	6F2J12	SEQ ID NO:45	6F2J12
SEQ ID NO:21	TrR3H1	SEQ ID NO:46	TrR3H1
SEQ ID NO:22	G6	SEQ ID NO:47	G6
SEQ ID NO:23	H8	SEQ ID NO:48	H8
SEQ ID NO:24	E7	SEQ ID NO:49	E7
SEQ ID NO:25	B6	SEQ ID NO:50	B6
SEQ ID NO:51	WT-APAO Esp001	SEQ ID NO:52	WT-APAO Esp001
SEQ ID NO:53	esp002C2	SEQ ID NO:54	esp002C2.pro
SEQ ID NO:55	esp002c3	SEQ ID NO:56	esp002C3.pro
SEQ ID NO:57	esp003C12	SEQ ID NO:58	esp003C12.pro
SEQ ID NO:59	RAT011C1	SEQ ID NO:60	rat011C1.pro
SEQ ID NO:61	RAT011C2	SEQ ID NO:62	rat011C2.pro
SEQ ID NO:63	RAT011C4	SEQ ID NO:64	RAT011C4
SEQ ID NO:65	APAO(B6)Glyc-	SEQ ID NO:66	APAO(B6)Glyc-(polypeptide)
SEQ ID NO:67	2E8	SEQ ID NO:68	6E9
SEQ ID NO:69	R3H1F	SEQ ID NO:70	2E8 (amino acid)
SEQ ID NO:71	6E9 (AMINO ACID)	SEQ ID NO:72	R3H1 (amino acid)
SEQ ID NO:73	Altered glycosylation site	SEQ ID NO:74	Nucleotides 1-15 of SEQ ID NO:1
SEQ ID NO:75	Silent variation of SEQ ID NO:74	SEQ ID NO:76	Amino Acids 1-5 of SEQ ID NO:26
SEQ ID NO:77	Amino Acids 1-26 of SEQ ID NO:26	SEQ ID NO:78	Conservative substitution variant of SEQ ID NO:77
SEQ ID NO:79	Conservative substitution variant of SEQ ID NO:77	SEQ ID NO:80	barley alpha amylase signal sequence DNA

SEQ ID NO:81	barley alpha-amylase type B isozyme mRNA	SEQ ID NO:82	translation of barley alpha amylase signal sequence
SEQ ID NO:83	translation of barley alpha amylase type B isozyme mRNA		
SEQ ID NO:84	1B5	SEQ ID NO:85	1B5
SEQ ID NO:86	4A9	SEQ ID NO:87	4A9
SEQ ID NO:88	4B11	SEQ ID NO:89	4B11
SEQ ID NO:90	4D11	SEQ ID NO:91	4D11
SEQ ID NO:92	5A3	SEQ ID NO:93	5A3
SEQ ID NO:94	5G10	SEQ ID NO:95	5G10
SEQ ID NO:96	6B11	SEQ ID NO:97	6B11
SEQ ID NO:98	6B3	SEQ ID NO:99	6B3
SEQ ID NO:100	7A5	SEQ ID NO:101	7A5
SEQ ID NO:102	7B8	SEQ ID NO:103	7B8
SEQ ID NO:104	7C10	SEQ ID NO:105	7C10
SEQ ID NO:106	7E9	SEQ ID NO:107	7E9
SEQ ID NO:108	9A2	SEQ ID NO109:	9A2
SEQ ID NO:110	9B10	SEQ ID NO:111	9B10
SEQ ID NO:112	9B8	SEQ ID NO:113	9B8
SEQ ID NO:114	9C5	SEQ ID NO:115	9C5
SEQ ID NO:116	9C7	SEQ ID NO:117	9C7
SEQ ID NO:118	9D3	SEQ ID NO:119	9D3
SEQ ID NO:120	9D9	SEQ ID NO:121	9D9
SEQ ID NO:122	9F2	SEQ ID NO:123	9F2
SEQ ID NO:124	9F3	SEQ ID NO:125	9F3
SEQ ID NO:126	9G10	SEQ ID NO:127	9G10

EXAMPLES

[0319] The following examples are illustrative and not limiting. One of skill will recognize a variety of non-critical parameters that can be altered to achieve essentially similar results.

Example I: Construction And Characterization Of Novel FD/FDD Molecules

[0320] Novel FD/FDD molecules were constructed which have altered enzymatic activity against FB1 and/or AP1 as compared to the activity of wild-type *E. spinifera* APAO against FB1 and/or AP1.

[0321] Molecules of FD/FDD were generated using methods described in patent applications and patents indicated *sic passim*, each of which is incorporated herein by reference in its entirety for all purposes. The new FD/FDD constructs displayed diverse amino acid and nucleotide differences from, *e.g.*, wild-type *E. spinifera* APAO (SEQ ID NOs:51-52) as well as displaying altered enzymatic parameters against FB1 and/or AP1. *See*, Figures 1, 2, 5, 7, and 8.

[0322] In order to mimic the conditions of maize apoplasts the enzymatic turnover experiments were performed in the following buffer at pH 5.5: 50 mM MES (K salt) (MES being 2-N-morpholino-ethane sulfonic acid), 2 mM Na_2PO_4, 1 mM NH_4NO_3, 1mM $CaCl_2$, and 1 mM $MgCl_2$. The enzyme was dialyzed after purification and quantification (by densitometry) into the above listed buffer and in the presence of 10 μM FAD (flavin-adenine-dinucleotide). After dialysis, the enzyme was diluted at ambient temperature to 5 ppm in the reaction buffer with 50 mM FB1 (Sigma Chemical Co., St. Louis , MO) and catalase (to degrade H_2O_2). The reactions were subsequently quenched with methanol and the quantity of remaining FB1 was measured using HPLC-Mass Spectrometry.

[0323] Kinetic parameters of the FD/FDD molecule constructs were determined by measuring the formation of hydrogen peroxide, a byproduct of FB1 and AP1 deamination (i.e., deamination caused by the FD/FDD molecules of the invention). In the presence of horseradish peroxidase, Amplex Red (Molecular Probes, Eugene, OR) reacts with hydrogen peroxide in a 1:1 stoichiometric ratio to produce a highly fluorescent molecule, resorufin, (oxidized Amplex red). Enzymatic rates for the FD/FDD constructs of the invention were measured as fluorescent units per minute (i.e., the fluorescence of oxidized Amplex Red formed). The kinetic parameters were determined graphically by evaluating the double

reciprocal of a rate vs. [substrate] plot, the Lineweaver-Burke reciprocal plot. For examples of exemplary kinetic parameters of FD/FDD molecules, *see* Figures 1, 2, and 8.

[0324] Additionally, the nucleic acid and amino acid make-up of the homologues were determined and compared. For example, see, Figure 3, which compares amino acid sequences from 2 exemplary homologues, R3H1 and B12, which share 8 amino acid changes from wild-type APAO (namely an alanine residue at position 118, a serine residue at position 136, a phenylalanine residue at position 209, a lysine residue at position 210, an isoleucine residue at position 237, a glutamic acid residue at position 272, a proline residue at position 274, and a glutamic acid residue at position 473). Furthermore, homologue R3H1 possesses a unique amino acid mutation at position number 193 (a change from asparagine to aspartic acid).

[0325] The enzymatic activity of homologue R3H1 was further characterized by determining its ability to reduce FB1 (*see*, Figure 5); its substrate specificity (*see*, Figure 6); its activity in transgenic maize calluses (either when cytosolicly expressed or when fused to a signal sequence) (*see*, Figure 7); its enzymatic parameters (*see*, Figure 8b); its FD/FDD activity over a range or pH (*see*, Figure 8c); and its thermostability (*see*, Figure 8d).

[0326] Additionally, homologues 2E8 and 6E9 were constructed (see, above) and were screened using in planta transient expression of secreted APAO enzyme followed by turnover of fumonisin B1 directly in the plant cell media and detection of the keto-FB1 reaction product by high throughput mass spectrometry.

[0327] These variants (i.e., 2E8 and 6E9) exhibited 6- and 10-fold increased production of keto FB1, respectively, relative to the homologue B6. Variant 6E9 contains 5 mutant amino acids present in homologues B6, E7, G6, and H8, a PCR-induced mutation resulting in a change from cysteine to arginine at amino acid position 425 and two silent mutations. Variant 2E8 has 2 mutant amino acids present in homologues B6, E7, G6, and H8, a PCR-induced change resulting in arginine at amino acid position 324 (the same position as one of the mutations in B6, E7, G6, and H8 but a different amino acid) and a PCR-induced mutation at amino acid position 384 resulting in substitution of glycine for glutamic acid.

Example II: Expression In Yeast And Selection Of Polynucleotides Exhibiting FD/FDD Activity

[0328] An exemplary homologue, R3H1 (SEQ ID NO:72), was truncated (SEQ ID NO:46) and verified for activity in *Pichia pastoris*. Truncated R3H1 (SEQ ID NO:46) was

truncated by 137 amino acid residues from the N-terminal of full length R3H1 (SEQ ID NO:72). The first amino acid residue of truncated R3H1 is a lysine rather than a proline (i.e., position 138 in full length R3H1 (SEQ ID NO:72)).

[0329] Polynucleotides of the present invention were inserted into yeast expression vector pPICZαA (Invitrogen) then transformed into *Pichia pastoris*. Variants picked by a Q-bot were placed into YPD (yeast extract, peptone and dextrose) containing zeocin in a 96-well format. The cultures were grown at 30°C, 275 rpm for 2 days. The cultures were then gridded on a 3x2 or 2x2 array, via Q-bot, onto a nylon membrane over a solid induction medium (1.5% Bacto-Agar, 0.5%, peptone, 4% biotin, 1.34% YNB, 400 mM MES pH 5.5 and 0.75% methanol). The cultures were induced for 2 days at 30°C, thus allowing for expression and secretion of molecules expressing fumonisin detoxification activity.

[0330] After induction for 2 days, the nylon membranes were transferred to an agarose reaction mix (1.5% agarose, 0.5 mg/ml Amplex Red, 80 U/ml horseradish peroxidase and 280μM fumonisin B1). Variants with equal or greater activity than the R3H1 homologue were transferred to YPD agar containing zeocin. Single colonies were selected and grown as described above. Membranes containing fumonisin detoxification expressing colonies were lifted off of the induction agar and incubated at 52°C for 45 minutes. These heat-treated colonies were then assayed on the above described agarose reaction mix.

[0331] After the incubation at 52°C, clones which displayed activity levels greater than homologue R3H1 were analyzed in liquid culture. The cultures were grown overnight at 29°C, 275 rpm in 10 ml YPD with zeocin followed by subculture into 20 ml of BMGY (phosphate buffered complex medium containing glycerol). After 1 day of growth at 29°C and 275 rpm, the cultures were spun down and washed once in a modified BMMH medium (MES pH 5.5, buffered minimal medium + histidine + 10μM FAD + 1% casamino acids) and resuspended in 2 ml of modified BMMH. The cultures were induced for 2 days and subsequently spun down, filtered and assayed using the Amplex Red coupled fluorescent assay for H_2O_2 (*see, infra*).

[0332] The resulting cultures were assayed for thermostability by pre-incubating the samples at 37°C for 10 minutes followed by assaying at room temperature. Fumonisin detoxification activity concentration was determined by coomassie staining and activities were adjusted to rfu/min/μg units (relative fluorescent units per minute per microgram). Four homologues were found to have similar activity to homologue R3H1, but greater thermostability. *See*, Figure 8a.

[0333] The k_{cat} and K_M for G6 were determined graphically with the Lineweaver-Burk plot and compared with R3H1. *See*, Figure 8b. Additionally the enzymatic activity of homologue G6 was determined over a range of pH. *See*, Figure 8c. The pH profiles (i.e., the profile of the enzymatic activity over a range of pH) of homologue R3H1 and homologue G6 are similar. Furthermore, the enzymatic activity of homologue G6 was determined after incubation at various temperatures for 10, 20, 30, and 45 minutes. For example, *see*, Figure 8d, which displays G6's activity at various temperatures following 10, 20, 30, and 45 minutes pre-incubation. The same manipulations are displayed for homologue R3H1 as well.

Example III: In Planta Turnover Of Fumonisin B1 By Exemplary Homologues Of The Invention

[0334] DNA constructs were made with selected homologues of the invention fused to a plant secretion signal. These constructs were used for transient expression of the selected FD/FDD polypeptides of the invention via *Agrobacterium tumefaciens* in *Nicotiana benthamiana* leaves.

[0335] Fumonisin B1 was injected with a syringe into the intercellular spaces of the expressing leaves. The fumonisin B1 was either labeled with ^{14}C or not labeled, depending on the method of detection. The leaf tissue was incubated at room temperature for three hours. Samples were then homogenized with 50% methanol, centrifuged and filtered. Conversion of fumonisin B1 to the oxidized keto-FB1 product was assessed by thin layer chromatography followed by autoradiography or by liquid chromatography followed by mass spectrometry. As seen in Figure 10, wild-type APAO converts very little (~5%) fumonisin B1 to keto-FB1. The B6 homologue of the invention (*see*, SEQ ID NO:25 and SEQ ID NO:50) converts at least ~98% of fumonisin B1 to keto-FB1. Similar results were also obtained with FD/FDD homologue E7 (*see*, SEQ ID NO:24 and SEQ ID NO:49). These results were confirmed using the liquid chromatography-mass spectrometry method (not shown). FD/FDD homologue R3H1 of the invention (*see*, SEQ ID NO:69 and SEQ ID NO:72) converted 80-90% of fumonisin B1 to keto-FB1 (not shown).

Example IV: In Planta Turnover Of FB1 In Maize

[0336] T-DNA expression constructs were made in which the full-length homologue R3H1(*see*, SEQ ID NO:69 and SEQ ID NO:72) sequence was modified in order to introduce the homologue B6 (*see*, SEQ ID NO: 25 and SEQ ID NO:50) mutations at cysteines 359 (C359->S) and 461 (C461->G). In addition, two amino acid substitutions to eliminate

potential glycosylation sites were engineered at amino acid 86 (N86->A; by means of A256->G and A257->G) and at amino acid 206 (S206->A, by means of A616->G, G616->C, and A628->G). The resulting open reading frame, designated APAO(B6) Glyc- (SEQ ID NO:65 and SEQ ID NO:66), was fused to a barley alpha amylase signal peptide and engineered into a T-DNA expression vector (designated PHP18303) for maize transformation *via Agrobacterium tumefaciens*. A second vector was prepared which lacked the signal peptide of PHP18303 (designated PHP18473). The promoter in both cases was the maize ubiquitin promoter and first intron, and the polyadenylation signal was from potato proteinase inhibitor II (PINII).

[0337] Immature maize embryos of genotype GS3 were excised at 9 days post pollination, co-cultivated with *Agrobacterium* LBA4404 cells harboring PHP18303 or PHP18473 for 2 days to allow for DNA transfer and transient expression of the FD/FDD homologue gene in outer cell layers of the embryos. At that point one set of embryos that had been incubated with each construct was removed for evaluation of total FD/FDD enzyme activity (i.e., here, the ability to degrade fumonisin B1) and ELISA protein following homogenization in 200 mM Na phosphate buffer pH 7 containing protease inhibitor cocktail, TWEEN-20 (0.01%) and 10 micromolar flavin adenine dinucleotide (FAD; Sigma-Aldrich).

[0338] The remaining embryos that had been co-cultivated with *Agrobacterium* containing the two constructs were transferred to 0.5 ml microfuge tubes containing 3 microliters of ^{14}C-labelled fumonisin B1 (0.3 mg/ml; approximately 1100 dpm per microgram) in 180 mM MES buffer, pH 5.5 containing 10 mM KCl and 0.2 mM $CaCl_2$, pH 5.5. The embryos were deposited scutellar side down in the liquid, the tube was sealed by capping, and allowed to incubate for 24 hours at 25°C.

[0339] After 24 hours, the bathing fluid was withdrawn from each tube and the entire amount (approximately 2.5 microliters) was spotted onto a reverse phase C18 thin-layer chromatography (TLC) plate, and plates were developed with MeOH:KCl (8:2). The embryos from the same incubation were then homogenized in 5 microliters of 50% methanol/water (to extract oxo-FB1 and at the same time inactivate any endogenous APAO activity (action against Fumonisin B1). This was accomplished using a sterile, plastic bacterial transfer loop whose loop end had been removed with a razor blade such that the blunt end matched the bottom radius of an 0.5 ml tube to allow effective maceration of the small tissue piece. The homogenate was centrifuged to pellet debris, and the entire supernatant fraction spotted onto reverse phase TLC plates and developed as described above. The relative position and amount of radioactivity per spot was measured by exposing the TLC

plate to a phosphorimager screen for 48 hours and then detecting and quantitating the phosporimage using a Molecular Dynamics STORM ™ system.

[0340] Both PHP18303 and PHP18473 co-cultivation resulted in roughly similar amounts of extractable protein and enzyme activity. For example, construct PHP18473 UBI-APAO(B6)Glyc-PINII had an ELISA reading of 26 ppm and an enzymatic activity level (i.e., percent of FB1 oxidized) of 19%, while construct PHP18303 UBI-BAA-APAO(B6)(Glyc-PINII had an ELISA reading of 45 ppm and an enzymatic activity level of 23%.

[0341] In the *in vivo* conversion portion of the experiment, oxo-FB1 product was detected in both supernatant and grindates of several of the embryos co-cultivated with PHP18303, but not in supernatants or grindates of embryos co-cultivated with PHP18473 (*see*, Figure 11). Figure 11 shows phosphorimage of thin-layer chromatogram from imbibed embryo supernatants (upper images) and extracts (lower images). On each panel, the leftmost spot is FB1 standard, and the rightmost spot is oxo-FB1 standard. The middle spots represent label in embryos 1 through 10 for each treatment. This indicates that although overall similar amounts of enzyme activity were generated by transient expression in both cases, only with a signal peptide present was enzyme capable of oxidizing exogenously-applied fumonisin substrate. Typically, only a few percent of cells in an embryo express a given transgene under these conditions, so the low percent conversion (relative to data obtained in tobacco leaves for a similar construct) is to be expected due to the large excess of substrate relative to enzyme.

[0342] Figure 12 illustrates the quantitation of radiolabel data from the TLC place in Figure 11. Percent conversion was initially calculated as percent of total label per embryo that was detected as oxo-FB1 (i.e., = counts in upper spots / [counts upper + counts lower] x 100). A similar calculation was made for a duplicate set of embryos co-cultivated with *Agrobacterium* LBA4404 cells containing a non-APAO T-DNA expression cassette (i.e., beta-glucuronidase), and the average of this "background" value was subtracted from each of the experimental percent conversion values for 18303 and 18473 co-cultivated embryos and supernatants.

[0343] The process for treating samples for the TLC assay and for the TLC assay itself comprised use addition of 200 µL extraction buffer (PBST, pH 7, 10 µM FAD, protease inhibitors (complete)) to each sample in Megatiter tubes. The samples had two 5/32" steel bearings added to each tube, which were capped tightly. Each sample was raptored for four cycles, 15 seconds each, with 5-10 minutes on ice between cycles. The samples were spun down and supernatants transferred to new tubes. The supernatants from the same constructs were pooled. The pellets were rinsed in 200 µL of 200 mM MES (pH 5.5) and spun down.

These supernatants were combined with the first supernatants (pooled). The pellets were again resuspended in 200 mM MES (pH 5.5) to make pellet suspension. About 200 µL from each sample were pooled (i.e., the ones from the same constructs were pooled) and spun down to rinse, followed by a resuspension in 200 µL of 200 mM MES (pH 5.5). The supernatants were filtered through a 0.2 µM SpinX units. The supernatants were concentrated in Microcon YM-10 units and the concentrates were resuspended to about 200 µL with 200 mM MES (pH 5.5) to assay via TLC. Storage was at –20°C.

[0344] The TLC assay for FD/FDD activity (also alternatively termed APAO activity in some instances herein) comprised incubation of 9 µL of each sample with 1 µL ^{14}C-FB1 (at about 10 mg/mL, in water) at room temperature for 2 days. 1 µL of such was spotted onto C18 TLC plates. Additionally, about 1 µg ^{14}C-FB1 and oxo-FB1 were used as controls. The plates were developed in MeOh:4% KCl (at an 8:2 ratio). The plates were exposed to phosphoscreen overnight and a Storm phosphoimager was used to read the results.

Example V: Expression of FD/FDD Homologue R3H1 in Transgenic Maize Callus

[0345] T-DNA expression constructs were made in which the full-length R3H1 homologue sequence was fused to a barley alpha amylase signal peptide at its N-terminus, and the resulting fusion engineered into a T-DNA expression vector (designated PHP17481) for maize transformation via *Agrobacterium tumefaciens*. A second vector was prepared which lacked the signal peptide of PHP17481 (designated PHP18473). Additional constructs were made using wild type APAO, either with a barley alpha amylase signal peptide N-terminal fusion (designated PHP17672) or without signal peptide (PHP17110). The promoter in all cases was the maize ubiquitin promoter and first intron (UBI), and the polyadenylation signal was from potato proteinase inhibitor II (PINII). All constructs contained the BAR gene for herbicide-based selection of transformed tissues on solid media. The signal peptide nucleotide and amino acid sequences used include:

a) barley alpha amylase signal sequence DNA which was fused in-frame upstream of the R3H1 open reading frame in PHPHP17490 and 17292 and which was obtained by synthesis based on published sequence of accession K02638, the sequence of which is:

ATG GCC AAC AAG CAC CTG TCC CTC TCC CTC TTC CTC GTG CTC CTC GGC CTC TCC GCC TCC CTC GCC TCC GGC (SEQ ID NO:80);

b) barley alpha-amylase type B isozyme mRNA, complete cds, clone pHV19, accession K02638, the sequence of which is:

ATG GCG AAC AAA CAC TTG TCC CTC TCC CTC TTC CTC GTC CTC CTT GGC CTG TCG GCC AGC TTG GCC TCC GGG (SEQ ID NO:81);

c) translation of barley alpha amylase signal sequence (which was fused in-frame upstream of R3H1 in PHPHP17490 and 17292), the sequence of which is:

MANKHLSLSLFLVLLGLSASLASG (SEQ ID NO:82);

d) translation of barley alpha-amylase type B isozyme mRNA, complete cds, clone pHV19, the sequence of which is:

MANKHLSLSLFLVLLGLSASLASG (SEQ ID NO:83).

[0346] Immature embryos of genotype GS3 were transformed with the above constructs via *Agrobacterium* co-cultivation, and stably-transformed callus was obtained by continuing herbicide selection on solid medium. FD/FDD expression level was evaluated in a minimum of five independent transformants per construct, and the line with the highest level off expression was chosen for further evaluation.

[0347] The amount of homologue protein present in each line was evaluated by an indirect ELISA assay employing polyclonal antisera raised in rabbits using wild-type APAO expressed in pGEX4t system (Amersham). The standard was APAO expressed in soluble pGEX4T1, which was subsequently GST-cleaved and purified according to manufacturers instructions. The results for such ELISA assay are as follows: PHP17110 (UBI-APAO-PINII) = 249 ppm; PHP17672 (UBI-BAA-APAO-PINII) = 94 ppm; PHP17481 (UBI-APAO(R3H1)-PINII) = 451 ppm; PHP17490 (UBI-BAA-APAO(R3H1)-PINII) = 170 ppm.

[0348] Equal weighed amounts of each callus lines were homogenized in phosphate buffered saline + Tween (pH 7.5) containing 10 uM flavin adenine dinucleotide cofactor (FAD; Sigma-Aldrich), and supernatants were filtered and concentrated using Microcon YM-10 ultrafiltration membranes (Amicon Corp.). Concentrates were reconstituted in MES buffer, pH 5.5 for R3H1-expressing callus, or phosphate buffer, pH 7.5, for wild type APAO-expressing callus. Following a second ultrafiltration step and reconstitution in the same buffer, extracts were assayed for fumonisin degrading activity using ^{14}C-fumonisin B1 (1.0 microgram per microliter final concentration, in the appropriate buffer; obtained from J. David Miller, Carleton Univ.) as a substrate in an overnight incubation at 25° C. Resolution of oxidized product from FB1 was accomplished by thin-layer chromatography on silica gel C_{18} plates using MeOH:4%KCl (8:2). The relative position and amount of radioactivity per spot was measured by exposing the TLC plate to a phosphorimager screen for 48 hrs and then

detecting and quantifying the resulting phosphorimage using a Molecular Dynamics STORM ™ system. Percent conversion of substrate to product was then calculated from the upper and lower spot values.

[0349] Figure 13 illustrates the fumonisin degrading activity in stably-transformed callus lines. Percent substrate oxidized was measured in a standard "APAO" assay utilizing ^{14}C-labeled FB1. The reaction mixture pH was optimized for the construct being evaluated, i.e., pH 7.0 for PHP17110 and PHP17672 (wild-type APAO), and pH 5.5 for PHP17481 and PHP17490 (R3H1). The supernatant activity (solid bars) and pellet activity (hatched bars) was determined separately for each extract. *See*, Figure 13.

[0350] FB1-oxidizing activity of calli transformed with R3H1 was higher than corresponding wild type APAO constructs, even when measured at the optimum pH for that enzyme (pH 7.5 for wild type; pH 5.5 for R3H1). Wild type APAO had undetectable activity when fused to a BAA signal sequence (construct PHP17672), which is in line with the low ELISA values for this callus line. The callus expressing R3H1 fused to BAA (PHP17490) had twice as much ELISA protein as wild type (PHP17672) (170 ppm), and it had much greater enzyme activity, particularly in the pellet fraction. While these data are from a single callus line in each case, they appear to represent the upper end of expression for each construct, since the highest expressing callus line in each case was chosen for evaluation. Therefore, maize-expressed shuffling variant R3H1 is active versus FB1, and retains significant activity on secretion, unlike the wild type APAO enzyme.

Example VI: APAO Assay of T0 Plants Transformed with clone 6e9 (SEQ ID NO: 71) for In Vitro and *In Vivo* APAO Activity

Sample Preparation

[0351] APAO variants 6e9 (SEQ ID NO:68) and 2e8 (SEQ ID NO:67), as full length open reading frames linked to barley alpha amylase signal sequence (BAA) (SEQ ID NO:80) were cloned into *Agrobacterium* expression vectors for maize transformation and used to transform GS3 x elite inbred F1 embryos. As a negative control, non-secreted, wild type APAO transformants were also generated. Transformation resulted in a total of 60 and 58 transformation events for 6e9 (SEQ ID NO:68) and 2e8 (SEQ ID NO:67), respectively.

[0352] For *in vitro* evaluation of transgene expression in the T0 generation, all T0 plants and 10 negative controls were processed and sampled for *in vitro* APAO activity. At the V5 to V6 plant stage (6 leaves, 4-5 completely unfurled), five leaf punches were taken

from the uppermost, completely unfurled leaf, transferred to megatiter tubes, and retained on ice. The leaf puncher was rinsed with 95% ethanol between samples. Extraction buffer (100mM PBST, pH7.5 + 10μM FAD + Complete (Roche) protease inhibitor cocktail) was added to each sample in a 400μL aliquot, along with two 5/32" steel bearings. The tubes were capped tightly and chilled on ice for 15 to 30 minutes. Samples were disrupted with a Geno/Grinder 2000 (SPEX CertiPrep, Metuchen, NJ) at 650 strokes/min for 15 seconds then chilled in ice water for 5 to 10 minutes. This process was repeated three more times. Extracts were spun down at 5000g at 4°C for 10 minutes.

TLC Assay of Extracts for APAO Activity

[0353] Nine microliters of each supernatant and one microliter of 14C-FB1 (10mg/mL, dH2O) were mixed in V-bottom microtiter plates such that [14C-FB1]= 1μg/μL, and wells were capped tightly. Samples were incubated overnight at room temperature. One microliter of each reaction was spotted onto TLC plates (Whatman KC18 Silica Gel 60A). Also ~1ug of 14C-FB1 and 14C-oxoFB1 (diluted in Extraction Buffer) was spotted on the ends of each plate. The plates were developed in MeOH:4% KCl (8:2) and air-dried. The plates were then exposed to a phosphor-screen overnight, and a Storm phosphorimager was used to obtain TLC images.

[0354] A total of 53 of the 60 T0 plants transformed with 6e9 were positive for APAO activity in this assay. A total of 54 of 58 T0 plants transformed with 2e8 were also positive for APAO activity.

Sampling and Processing of Leaf Tips for In Vivo Conversion Studies

[0355] Three of the positive plants (28421745, 28421778, 28421777) at stage V7 (7 leaves, 5-6 completely unfurled) and one negative control (28421798) were selected. The terminal ~10cm was removed from the tip of the most recently fully-opened (6th) leaf and transferred to a 50mL falcon tube containing ~10mL of dH2O. Leaf tips were rinsed under dH2O to remove debris, pollen, etc. The bottom of each leaf tip was re-cut underneath dH2O (to prevent cavitation) to ~5-6cm, and transferred to new tubes containing ~10mL dH2O. Leaves were soaked for ~15 minutes, the dH2O was changed, and the process was repeated two more times (to remove any enzyme that may have leaked out of injury site). Leaf tips were then transferred to 7mL scintillation vials containing 450μL of dH2O. 50μL of 14C-FB1 (10mg/mL, dH2O) was added to each sample such that final [14C-FB1]= 1ug/uL. Each vial was partially sealed with parafilm and the vials were placed in a rack in a crisper lined

with moist paper towels, ensuring a tight lid. Samples were incubated for 72 hours on the lab bench at room temperature, 4 to 6 inches below 24-hour fluorescent lights.

[0356] Toxin solutions were transferred to new tubes at t=72 hours and 1μL and 2μL of the ^{14}C-FB1 solutions were spotted onto TLC plates (Whatman KC18 Silica Gel 60A). Also ~1ug of ^{14}C-FB1 and ^{14}C-oxoFB1 were spotted on the ends of each plate. Plates were developed in MeOH:4% KCl (8:2) and air-dried. Plates were exposed to phosphor-screen overnight, and a Storm phosphorimager was used to get TLC images.

[0357] Leaf tips were rinsed three times in dH_2O. The bottom ~1cm of each leaf that was dipped in the toxin solution was removed. Leaf tips were then transferred to 13mm glass tubes, frozen in liquid nitrogen and ground to a powder with a glass rod. 500μL of 50% ACN (in dH_2O) was added to each powdered sample and ground further with a glass rod. Grindates were transferred to 2mL tubes, and the glass tubes were rinsed with another 500μL of 50% ACN (in dH_2O), and pooled with the first grindate. Tubes holding the samples were then sonicated in a water bath for 15 minutes and spun down at 10,000g at 4°C for 10 minutes to pellet any debris. Supernatant was transferred to new tubes. The powder was re-extracted (sonicated) with another 500uL of 50% ACN (in dH_2O) as before, and supernatant was pooled with first supernatant. Supernatants were then filtered through 0.2μM nylon SpinX units.

Determination Of DPM's (Disintegrations Per Minute)

[0358] 20uL of each extract supernatant was mixed with ~6mL scintillation fluid. External standard quench correction was used to determine dpm's in each sample. The concentration of ^{14}C-label in each sample was estimated based on the activity of ^{14}C-FB1 (~946dpm/ug ^{14}C-FB1). The volume of each extract that would yield ~20ug of ^{14}C-label (to make 20uL of ~1ug/uL stocks for TLC assays) was determined. The calculated volumes were then dried down in the SpeedVac. Residues were dissolved in 20μL of MeOH to get ~1ug/uL of ^{14}C-label.

[0359] 1μL and 2μL of each ~1ug/uL sample were spotted onto TLC plates (Whatman KC18 Silica Gel 60A). Also ~1ug of ^{14}C-FB1 and ^{14}C-oxoFB1 were spotted on the ends of each plate. The plates were developed in MeOH:4% KCl (8:2) and air-dried. Plates were exposed to phosphor-screen overnight, and a Storm phosphorimager was used to get TLC images. ImageQuant software was used to determine the volume of counts for each spot, and % conversion of FB1 to oxoFB1 was estimated in each sample.

[0360] All three APAO-expressing T0 generation plants expressing 6e9 (SEQ ID NO: 68) which were tested showed significant conversion of labeled FB1 to oxo-FB1 (estimated at 50% to 60% conversion). The three APAO-expressing T0 generation plants expressing 2e8 (SEQ ID NO: 70) which were tested showed conversion ranging from 67% to 80% of FB1 to oxo-FB1. The non-transformed control extracts showed no detectable oxo-FB1. Only trace amounts of oxo-FB1 were detected in the remaining leaf imbibition buffer, indicating that conversion occurred *in vivo* and not as a result of enzyme leakage into the imbibition solution followed by uptake of oxidized FB1.

Example VII. *Agrobacterium*-mediated Transformation

[0361] For *Agrobacterium*-mediated transformation of maize with an FD/FDD nucleotide sequence of the invention, operably linked to a promoter, preferably the method of Zhao is employed (U.S. Patent No. 5,981,840, and PCT patent publication WO98/32326; the contents of which are hereby incorporated by reference). Briefly, immature embryos are isolated from maize and the embryos contacted with a suspension of *Agrobacterium*, where the bacteria are capable of transferring the DNA construct containing the nucleotide sequence of the invention to at least one cell of at least one of the immature embryos (step 1: the infection step). In this step the immature embryos are preferably immersed in an *Agrobacterium* suspension for the initiation of inoculation. The embryos are co-cultured for a time with the *Agrobacterium* (step 2: the co-cultivation step). Preferably the immature embryos are cultured on solid medium following the infection step. Following this co-cultivation period an optional "resting" step is contemplated. In this resting step, the embryos are incubated in the presence of at least one antibiotic known to inhibit the growth of *Agrobacterium* without the addition of a selective agent for plant transformants (step 3: resting step). Preferably the immature embryos are cultured on solid medium with antibiotic, but without a selecting agent, for elimination of *Agrobacterium* and for a resting phase for the infected cells. Next, inoculated embryos are cultured on medium containing a selective agent and growing transformed callus is recovered (step 4: the selection step). Preferably, the immature embryos are cultured on solid medium with a selective agent resulting in the selective growth of transformed cells. The callus is then regenerated into plants (step 5: the regeneration step), and preferably calli grown on selective medium are cultured on solid medium to regenerate the plants.

Example VIII: Soybean Embryo Transformation

[0362] Soybean embryos are bombarded with a plasmid containing an FD/FDD nucleotide sequence of the instant invention operably linked to a ubiquitin promoter as follows. To induce somatic embryos, cotyledons, 3-5 mm in length dissected from surface-sterilized, immature seeds of the soybean cultivar A2872, are cultured in the light or dark at 26°C on an appropriate agar medium for six to ten weeks. Somatic embryos producing secondary embryos are then excised and placed into a suitable liquid medium. After repeated selection for clusters of somatic embryos that multiplied as early, globular-staged embryos, the suspensions are maintained as described below.

[0363] Soybean embryogenic suspension cultures can maintained in 35 ml liquid media on a rotary shaker, 150 rpm, at 26° C with florescent lights on a 16:8 hour day/night schedule. Cultures are subcultured every two weeks by inoculating approximately 35 mg of tissue into 35 ml of liquid medium.

[0364] Soybean embryogenic suspension cultures may then be transformed by the method of particle gun bombardment (Klein *et al.* (1987) *Nature* (London) *327*:70-73, U.S. Patent No. 4,945,050). A DuPont Biolistic PDS1000/HE instrument (helium retrofit) can be used for these transformations.

[0365] A selectable marker gene that can be used to facilitate soybean transformation is a transgene composed of the 35S promoter from Cauliflower Mosaic Virus (Odell *et al.* (1985) *Nature 313*:810-812), the hygromycin phosphotransferase gene from plasmid pJR225 (from *E. coli*; Gritz *et al.* (1983) *Gene 25*:179-188), and the 3' region of the nopaline synthase gene from the T-DNA of the Ti plasmid of *Agrobacterium tumefaciens*. The expression cassette comprising the FD/FDD sequence operably linked to the ubiquitin promoter can be isolated as a restriction fragment. This fragment can then be inserted into a unique restriction site of the vector carrying the marker gene.

[0366] To 50 µl of a 60 mg/ml 1 µm gold particle suspension is added (in order): 5 µl DNA (1 µg/µl), 20 µl spermidine (0.1 M), and 50 µl $CaCl_2$ (2.5 M). The particle preparation is then agitated for three minutes, spun in a microfuge for 10 seconds and the supernatant removed. The DNA-coated particles are then washed once in 400 µl 70% ethanol and resuspended in 40 µl of anhydrous ethanol. The DNA/particle suspension can be sonicated three times for one second each. Five microliters of the DNA-coated gold particles are then loaded on each macro carrier disk.

[0367] Approximately 300-400 mg of a two-week-old suspension culture is placed in an empty 60x15 mm petri dish and the residual liquid removed from the tissue with a pipette.

For each transformation experiment, approximately 5-10 plates of tissue are normally bombarded. Membrane rupture pressure is set at 1100 psi, and the chamber is evacuated to a vacuum of 28 inches mercury. The tissue is placed approximately 3.5 inches away from the retaining screen and bombarded three times. Following bombardment, the tissue can be divided in half and placed back into liquid and cultured as described above.

[0368] Five to seven days post bombardment, the liquid media may be exchanged with fresh media, and eleven to twelve days post-bombardment with fresh media containing 50 mg/ml hygromycin. This selective media can be refreshed weekly. Seven to eight weeks post-bombardment, green, transformed tissue may be observed growing from untransformed, necrotic embryogenic clusters. Isolated green tissue is removed and inoculated into individual flasks to generate new, clonally propagated, transformed embryogenic suspension cultures. Each new line may be treated as an independent transformation event. These suspensions can then be subcultured and maintained as clusters of immature embryos or regenerated into whole plants by maturation and germination of individual somatic embryos.

Example IX: Transformation and Regeneration of Transgenic Plants

[0369] Immature maize embryos from greenhouse donor plants are bombarded with a plasmid containing the FD/FDD sequences of the present invention operably linked to a ubiquitin promoter and the selectable marker gene PAT (Wohlleben *et al.* (1988) *Gene 70*:25-37), which confers resistance to the herbicide Bialaphos. Alternatively, the selectable marker gene is provided on a separate plasmid. Transformation is performed as follows. Media recipes follow below.

Preparation of Target Tissue

[0370] The ears are husked and surface sterilized in 30% Clorox bleach plus 0.5% Micro detergent for 20 minutes, and rinsed two times with sterile water. The immature embryos are excised and placed embryo axis side down (scutellum side up), 25 embryos per plate, on 560Y medium for 4 hours and then aligned within the 2.5-cm target zone in preparation for bombardment.

Preparation of DNA

[0371] This plasmid DNA plus plasmid DNA containing a PAT selectable marker is precipitated onto 1.1 μm (average diameter) tungsten pellets using a $CaCl_2$ precipitation procedure as follows:

100 µl prepared tungsten particles in water

10 µl (1 µg) DNA in Tris EDTA buffer (1 µg total DNA)

100 µl 2.5 M $CaC1_2$

10 µl 0.1 M spermidine

[0372] Each reagent is added sequentially to the tungsten particle suspension, while maintained on the multitube vortexer. The final mixture is sonicated briefly and allowed to incubate under constant vortexing for 10 minutes. After the precipitation period, the tubes are centrifuged briefly, liquid removed, washed with 500 µl 100% ethanol, and centrifuged for 30 seconds. Again the liquid is removed, and 105 µl 100% ethanol is added to the final tungsten particle pellet. For particle gun bombardment, the tungsten/DNA particles are briefly sonicated and 10 µl spotted onto the center of each macrocarrier and allowed to dry about 2 minutes before bombardment.

Particle Gun Treatment

[0373] The sample plates are bombarded at level #4 in particle gun #HE34-1 or #HE34-2. All samples receive a single shot at 650 PSI, with a total of ten aliquots taken from each tube of prepared particles/DNA.

Subsequent Treatment

[0374] Following bombardment, the embryos are kept on 560Y medium for 2 days, then transferred to 560R selection medium containing 3 mg/liter Bialaphos, and subcultured every 2 weeks. After approximately 10 weeks of selection, selection-resistant callus clones are transferred to 288J medium to initiate plant regeneration. Following somatic embryo maturation (2-4 weeks), well-developed somatic embryos are transferred to medium for germination and transferred to the lighted culture room. Approximately 7-10 days later, developing plantlets are transferred to 272V hormone-free medium in tubes for 7-10 days until plantlets are well established. Plants are then transferred to inserts in flats (equivalent to 2.5" pot) containing potting soil and grown for 1 week in a growth chamber, subsequently grown an additional 1-2 weeks in the greenhouse, then transferred to classic 600 pots (1.6 gallon) and grown to maturity.

Bombardment and Culture Media

[0375] Bombardment medium (560Y) comprises 4.0 g/l N6 basal salts (SIGMA C-1416), 1.0 ml/l Eriksson's Vitamin Mix (1000X SIGMA-1511), 0.5 mg/l thiamine HCl, 120.0 g/l sucrose, 1.0 mg/l 2,4-D, and 2.88 g/l L-proline (brought to volume with D-I H_20 following adjustment to pH 5.8 with KOH); 2.0 g/l Gelrite (added after bringing to volume with D-I H_20); and 8.5 mg/l silver nitrate (added after sterilizing the medium and cooling to room temperature). Selection medium (560R) comprises 4.0 g/l N6 basal salts (SIGMA C-1416), 1.0 ml/l Eriksson's Vitamin Mix (1000X SIGMA-1511), 0.5 mg/l thiamine HCl, 30.0 g/l sucrose, and 2.0 mg/l 2,4-D (brought to volume with D-I H_20 following adjustment to pH 5.8 with KOH); 3.0 g/l Gelrite (added after bringing to volume with D-I H_20); and 0.85 mg/l silver nitrate and 3.0 mg/l bialaphos(both added after sterilizing the medium and cooling to room temperature).

[0376] Plant regeneration medium (288J) comprises 4.3 g/l MS salts (GIBCO 11117-074), 5.0 ml/l MS vitamins stock solution (0.100 g nicotinic acid, 0.02 g/l thiamine HCL, 0.10 g/l pyridoxine HCL, and 0.40 g/l glycine brought to volume with polished D-I H_20) (Murashige and Skoog (1962) *Physiol. Plant. 15*:473), 100 mg/l myo-inositol, 0.5 mg/l zeatin, 60 g/l sucrose, and 1.0 ml/l of 0.1 mM abscisic acid (brought to volume with polished D-I H_20 after adjusting to pH 5.6); 3.0 g/l Gelrite (added after bringing to volume with D-I H_20); and 1.0 mg/l indoleacetic acid and 3.0 mg/l bialaphos (added after sterilizing the medium and cooling to 60°C). Hormone-free medium (272V) comprises 4.3 g/l MS salts (GIBCO 11117-074), 5.0 ml/l MS vitamins stock solution (0.100 g/l nicotinic acid, 0.02 g/l thiamine HCL, 0.10 g/l pyridoxine HCL, and 0.40 g/l glycine brought to volume with polished D-I H_20), 0.1 g/l myo-inositol, and 40.0 g/l sucrose (brought to volume with polished D-I H_20 after adjusting pH to 5.6); and 6 g/l bacto-agar (added after bringing to volume with polished D-I H_20), sterilized and cooled to 60° C.

[0377] While the foregoing invention has been described in some detail for purposes of clarity and understanding, it will be clear to one skilled in the art from a reading of this disclosure that various changes in form and detail can be made without departing from the true scope of the invention. For example, all the techniques and apparatus described above may be used in various combinations. All publications, patents, patent applications, and/or other documents cited in this application are incorporated by reference in their entirety for all purposes to the same extent as if each individual publication, patent, patent application, and/or other document were individually indicated to be incorporated by reference for all purposes.